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Arbuscular mycorrhizal research in coastal sand dunes : A review

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Abstract

Coastal sand dunes are extreme habitats with special reference to salinity, drought and disturbance. A variety of plant species and microorganisms have been adapted to such ecosystem. Arbuscular mycorrhizal (AM) fungi are known for their wide distribution in coastal sand dunes throughout the world. Besides facilitating the uptake of nutrient by plants, AM fungi play a vital role in succession of dune vegetation and formation of stable sand aggregates. Majority of the studies on coastal sand dune AM fungi and their application in sand dune restoration have been confined to temperate regions. Inventory and AM fungal application in tropical coastal sand dune restoration and stabilization are yet to be intensified. The present review has focused on : geographical distribution, species richness, diversity, dormancy and survival of AM fungi in coastal sand dunes; impact of sand dune edaphic features and disturbance on AM fungal activities; role of AM fungi in dune plant succession, dune restoration and stabilization; pattern of formation of wind-resistant sand aggregates and application of AM fungi in sand dune stabilization. The importance and future outlook of AM fungi in coastal sand dunes is also discussed.

(**Key words** : dune vegetation/succession/rhizosphere/sand aggregation/dune disturbance/edaphic features/arbuscular mycorrhizae/geographical diversity/species richness/endophytes).

Introduction

Coastal sand dunes are the low nutrient and unstable habitats of vital economic and ecological importance[1,2]. Despite the geographical differences, coastal sand dunes have been considered as a specific ecosystem due to several common environmental features. The major limiting factors leading to the loss and instability of coastal sand dune ecosystem are : shoreline regression, salt spray, sandy texture and abrasion, sand accretion and erosion, low moisture and nutrient availability, high soil temperatures, herbivory and human interference[3]. Arbuscular mycorrhizal (AM) fungal associations have a wide range of habitats : aquatic, deserts, coastal sand dunes, tropical rain forests and canopy epiphytes[4]. Although the research efforts from 1970 onwards concentrated mainly on the occurrence of AM fungi in field conditions[5], knowledge on functioning of AM fungi in the field is limited[6,7] In field studies the main focus was on agricultural ecosystems, whereas the natural ecosystems and disturbed ecosystems have been neglected[8]. The AM fungi are widely distributed biota in coastal sand dunes throughout the world[9]. They play an important role in plant succession and stabilization of maritime sand dunes by facilitating nutrient uptake, binding sand grains into stable aggregates and improving soil structure[10].

The present review addresses the role of AM fungi in coastal sand dunes. The major subtopics considered for discussion include : inventory in different continents; species richness and diversity; seasonal periodicity; sand aggregation and dune stability; role in the establishment and succession of maritime vegetation; spore dormancy and germination; spore ageing, parasitism and death; effect of edaphic factors; impact of disturbance; effect of endophytes; AM fungal status of non-mycorrhizal and facultatively mycorrhizal plant species.

Inventory

Inventory is the first step to understand the role of microorganisms in an ecosystem. Coastal sand dunes are favourable for the establishment and functioning of AM fungi mainly because of their low phosphorus content[2,11]. Ever since the importance of AM fungi in sand dune succession has been recognized[12], many surveys have been conducted in temperate, subtropical and tropical coastal sand dunes of the world.

Temperate locations :

Inventory of AM fungi in coastal sand dunes of temperate locations were mainly confined to the United States and Europe. More attention has been focused on the

Atlantic coast of United States. In Rhode Island, *Ammophila breviligulata* (American beach grass) dominates the dune vegetation and exhibits greatest spore density in the rhizosphere[11]. Studies on the spore population of five species of AM fungi in coastal sand dunes of Massachusetts revealed the antagonistic interactions between them[13]. Twenty three species of AM fungi were recovered from the major plant species growing on barrier dunes along 355 km coastal stretch extending from northern New Jersey (*A. breviligulata*) to Virginia (*A. breviligulata, Myrica pensylvanica, Solidago sempervirens* and *Uniola paniculata*)[14]. Studies on six locations along the transect showed an increase in overall mean species richness of AM fungi in all plant species from north to south. Several new species of AM fungi have been described from the sand dunes of temperate locations of the Atlantic and Pacific coasts (Table 1). *Gigaspora gigantea* is the most dominant AM fungal species in the sand dunes of Atlantic coast of United States[15].

Table 1.– New species of arbuscular mycorrhizal fungi described from the maritime sand dunes.

Taxon	Location	Reference
Acaulosporaceae		
Acaulospora elegans Trappe & Gerd.	Orgeon, USA	110
A. gadanskensis Balszkowski	Chalupy, Poland	111
A. koskei Balszkowski	Slowinski, Poland	112
A. polonica Balszkowski	Hel, Poland	111
Entrophospora baltica Balszkowski, Madej & Tadych	Northwest, Poland	113
Gigasporaceae		
Scutellospora arenicola Koske & Halvorson	California, USA	114
S. coralloidea (Trappe, Gerd. & Ho) Walker & Sanders	Oregon, USA	110
S. dipapillosa (Walker & Koske) Walker & Sanders	Rhode Island, USA	115

Table 1, Contd...

S. erythropa (Koske & Walker) Walker & Sanders	Rhode Island, USA	21
S. fulgida Koske & Walker	Virginia, USA	116
S. hawaiiensis Koake & Gemma	Kaua'i, Hawaii	79
S. pellucida (Nicol. & Schenck) Walker & Sanders	Florida, USA	116
S. persica (Koske & Walker) Walker & Sanders	New Jersey, USA	115
S. reticulata (Koske, Miller & Walker) Walker & Sanders	Rhode Island, USA	117
S. verrucosa (Koske & Walker) Walker & Sanders	Virginia, USA	115
S. weresubiae Koske & Walker	Virginia, USA	116
Glomaceae		
Glomus corymbiforme Balszkowski	Swinoujscie, Poland	118
G. gibbosum Balszkowski	Swinoujscie, Poland	119
G. globiferum Koske & Walker	New Jersey, USA	120
G. halon Rose & Trappe	Lincolnshire, England	121
G.microaggregatum Koske, Gemma & Olexia	California, USA	122
G. microcarpum Tulasne & Tulasne	Idaho and California, USA	110
G. minutum, Balszkowski, Tadych & Madej	Northwest, Poland	123
G. monosporum Gerd. & Trappe	Oregon, USA	110
G. nanolumen Koske & Gemma	Kaua'i, Hawaii	124
G. pallidum Hall	Tautuku, New Zealand	125
G. pansihalos Berch & Koske	California, USA	126
G. pustulatum Koske, Friese, Walker & Dalpe	Rhode Island, USA	127
G. trimurales Koske & Halvorson	California, USA	114

Studies on European coastal sand dunes confined mainly to Italy and Poland. On the Italian dunes, members of the family Asteraceae, Papilionaceae and Poaceae were heavily colonized[16,17]. Two plant species belonging to the family Caryophyllaceae were also colonized by AM fungi in stable sand dunes of Italy[16]. The well-fixed dunes at Migliarino, Italy showed varied AM fungal flora similar to that in the dunes of Australia[18] and Rhode Island[11]. A significant correlation was found between spore density and extent of root colonization. Maritime sand dunes of Poland yielded several new and interesting species of AM fungi (Table 1).

Subtropical locations :

Sand dunes of many subtropical locations were surveyed for the existence and activity of AM fungi. Such locations include : Florida, Mexico, Baja California, Brazil, Japan, Australia and Pakistan. Root samples of 37 plant species, along the succession gradient of mobile to stable dunes of the Gulf of Mexico revealed the colonization of 97% plant species by AM Fungi[19]. The mycorrhizal inoculum potential increased with dune stabilization. Plant species belonging to the so-called non-mycorrhizal families (*Aizoaceae* and *Cyperaceae*) were also colonized by AM fungi. Root colonization gets initiated during the rainy season and as the soil dries, arbuscules decline and vesicles begin to increase as observed in the dunes of Baja California[20]. The phenology of plant species and fungal species coincides. *Scutellospora erythropa* described from Bahamas was common in sand dunes of North America[21]. *Acaulospora scrobiculata* was the most abundant species, while *Gigaspora albida* was the most frequent species in Brazilian dunes[9].

In Japanese coastal sand dunes, Asai[22] first encountered AM fungi colonization in dune plants. A total of six species of AM fungi was recorded from Japanese dunes[23]. Among them *Glomus* spp. were most dominant. The highest density of spores was found to be associated with the rhizomes and the spores were detected up to a depth of 130 cm. Logan *et al.*[24] studied 41 plant species along the coast of New South Wales, Australia. Out of them 36 species were mycorrhizal. Interesting feature was that the members of the so-called non-mycorrhizal family (*Aizoaceae, Brassicaceae, Cyperaceae* and *Chenopodiaceae*) were also colonized by AM fungi. Koske[18] studied the coastal sand dune vegetation at New South Wales at different stages of succession, which revealed the greater spore density in stabilized old dunes than in recent and mobile dunes. Among rest of the subtropical locations, coastal sand dunes of Pakistan revealed the occurrence of only one kind of AM fungus, its spore density ranging between 0.01 and 0.3/g[25].

Tropical locations :

Compared to temperate and subtropical locations, very few surveys are available on AM fungi of maritime sand dunes of tropical region. Relatively the sand dunes and vegetation of Hawaiin Islands have been investigated more intensively. Hasain Islands got erupted from the sea nearly 30 million years ago. It was exposed to continuous volcanic activities and sand dune formation[26]. These islands consist of many sand dune habitats, which are in the early stages of primary succession. The volcanic activities resulted in continuous selection of vegetation where mycotrophy played an important role. Most of the seedlings grown on the drift line area were associated with AM fungi[27]. Roots of 23 out of 31 vascular plant species showed AM fungal colonization. Mycorrhizal index was highest in 12 plant species[27]. Koske[28] screened eight sand dune plant species and recorded 12 species of AM fungi. *Sclerocystis sinuosa* and *Glomus microaggregatum* were dominant. A survey of sixteen plant species from the dunes of Kaua'i, Hawaii revealed the occurrence of 14 species of AM fungi[29]. *Scutellospora hawaiiensis* was the common species. Dead and parasitized spores outnumbered the live AM spores.

A few surveys are available from the Indian subcontinent. Along the coast of Chennai covering 36 km transect, 56 plant species were surveyed by Mohankumar *et al.*[30]. The rhizosphere of all plant species possessed AM fungi, but roots of only 35 plant species had AM fungal colonization. Among the members of the family Cyperaceae, *Cyperus stoloniferous* colonized by AM fungi, while *Kyllinga brevifolia* was not colonized. The rhizosphere of 12 plant species established on the west coast of India in post-monsoon season yielded the spores of 16 species of AM fungi[31]. Colonization of roots ranged from 34 to 80 percent. Spores of *Scutellospora gregaria* was most abundant, while *Glomus albidum* was most frequent. On non-vegetated dunes, spores of five species were recovered, among them *Glomus albidum* and *G. lacteum* were dominant. Two years seasonal survey of AM fungi associated with *Ipomoea pes-caprae* and *Launaea sarmentosa* on the sand dunes of west coast of Karnataka revealed the occurrence of 41 and 28 species respectively[32,33]. Colonization of roots of AM fungi was at a peak during post-monsoon season, but the spore density was maximum during summer (*I. pes-caprae*) or monsoon (*L. sarmentosa*). The AM fungal phenology (colonization, spore density and species richness) was coincided with the plant phenology which might have increased the fitness of *I. pes-caprae* and *L. sarmentosa* on the coastal sand dunes. The diversity of AM fungi associated with *I. pes-caprae* on 10 sand dunes at a stretch of 100 km of west coast of India consist of moderately disturbed dunes (MDD) and severely disturbed dunes (SDD) were studied

during wet and dry seasons by Beena *et al.*[34]. The vegetation cover, AM colonization, spore richness and diversity were greater in MDD than in SDD irrespective of the season. The AM fungal richness and spore density was strongly correlated with rhizosphere nitrogen. *Glomus mosseae, G. dimorphicum* and *Gigaspora gigantea* were most common in both MDD and SDD.

A survey conducted on the plant species of coastal land of Singapore after five years of reclamation did not show the presence of AM fungi[35]. Non-mycorrhizal plant species were prevalent in reclaimed area. The success and persistence of non-mycorrhizal plant species on reclaimed area was due to the lack of AM propagules and competition from mycorrihazal plant species. A gradual increase in colonization of mycorrhizal plant species away from the reclaimed area was seen, the rhizosphere of *Casuarina equisitifolia* consists of *Gigaspora rosea* and *Rhynchelytrum repens* and *Desmodium heterophyllum* consist of *Glomus macrocarpum.*

Comparison of AM Flora

Distribution :

Comparison of AM flora on different sand dunes by Koske and Tews[36] revealed a maximum similarity between the Rhode Island and New Jersey to Virginia. Although the AM fungi have a broad host range, the observed differences in similarity might be due to the independent responses of beach grasses, AM fungi and a set of ecological factors[36]. The AM fungal community of Hawaiian Islands is very distinct from that of Australia, Atlantic and Pacific coasts of United States, Scotland and Italy[28]. So far about the 76 species of AM fungi have been recorded form the coastal sand dunes (Table 2). Relatively more studies have been conducted from the sand dunes of temperate regions than the tropical and subtropical regions. Due to this disparity, comparison of species richness and diversity between the wide geographical regions may not be relevant. From Table 2 it can be deduced that 49, 38 and 23 species have been reported from temperate, tropical and subtropical regions respectively. Out of the 76 species, 11 species (*Acaulosora scrobiculata, Gigaspora albida, G. gigantea, Scutellospora calospora, S. erythropa, S. gregaria, S. pellucida, Glomus aggregatum, G. constrictum, G. fasciculatum* and *G. globiferum*) are cosmopolitan in distribution. *Acaulospora scrobiculata* is frequent in south-eastern Australia, Brazil and Atlantic dunes of United States. *Gigaspora gigantea* and *Glomus aggregatum* are common in east coast of North America. *Scutellospora erythropa* is a common member of the sand dunes of Atlantic coast of the United States. *Glomus fasciculatum* was encountered by Kulkarni *et al.*[31] in the west coast of India, it is also widely distributed

in California to British Columbia. *Gigaspora albida* and *Scutellospora erythropa* were more frequent and *Gigaspora globiferum* and *S. calospora* were frequent in the west coast of India[31-34]

Table 2.– Geographical distribution of arbuscular mycorrhizal fungi on coastal sand dunes.

(**Temperate locations*—(A) North America : 11, 13, 14, 21, 45, 68, 83, 110, 114, 122, 127, 128, 129. (B) Europe : 16, 17, 38, 110, 112, 113, 118, 119, 121, 123, 129. (C) Japan : 23, 41.

Subtropical locations—(D) Australia : 18, 125, 129, 130. (E) Florida : 40, 58, 128, 131. (F) Brazil : 9.

Tropical locations—(G) Hawaiian Islands : 28, 29, 79. (H) India : 30–32, 34. (I) Singapore : 35).

Taxon	Location*
Acaulosporaceae	
Acuaulospora elegans Trappe & Gerd	A
A. denticulata Sieverding & Toro	H
A. gadanskensis Balszkowski	B
A. koskei Balszkowski	B
A. lacunosa Morton	A
A. laevis Gerd. & Troppe	A, B, D, E
A. mellea Spain & Schenck	A
A. morrowiae Spain & Schenck	E
A. nicolsonii Walker, Reed & Sander	H
A. polonica Balszkowski	B
A. scrobiculata Trappe	A, D-G
A. spinosa Walker & Trappe	H
A. taiwanica Hu	H
Entrophospora baltica Balszkowski, Madej & Tadych	B

Table 2, Contd...

E. infrequens (Hall) Ames & Schneider	A, H
E. schenckii Sieverding & Toro	H
Gigasporaceae	
Gigaspora albida Schenck & Smith	A, E, F, H
G. decipens Hall & Abbott	H
G. gigantea (Nicol. & Gerd.) Gerd. & Trappe	A, D, E, H
G. gregaria (Schenck & Nicolson) Walker & Sanders	H
G. margarita Becker & Hall	A, H
G. rosea Nicolson & Schenck	A, I
Scutellospora arenicola Koske & Halvorson	A, H
S. calospora (Nicol. & Gerd.) Walker & Sanders	A, B, D, H
S. coralloidea (Trappe, Gerd. & Ho) Walker & Sanders	A, F
S. dipapillosa (Walker & Koske) Walker & Sanders	A, E
S. erythropa (Koske & Walker) Walker & Sanders	A, E, H
S. fulgida Koske & Walker	A, E
S. gilmorei (Trappe & Gerd.) Walker & Sanders	A
S. gregaria (Schenck & Nicolson) Walker & Sanders	A, C, F-I
S. hawaiiensis Koske & Gemma	G
S. nigra (Redhead) Walker & Sanders	H
S. pellucida (Nicol. & Schenck) Walker & Sanders	A, E, H
S. persica (Koske & Walker) Walker & Sanders	A, B
S. reticulata (Koske, Miller & Walker) Walker & Sanders	A
S. scutata Walker & Diederichs	F

Table 2, Contd..

S. verrucosa (Koske & Walker) Walker & Sanders	A, E
S. weresubiae Koske & Walker	A, E, F
Glomaceae	
Glomus aggregatum schenck & Smith	A, C, E, G, H
G. albidum Walker & Rhodes	H
G. caledonium (Nicol. & Gerd.) Trappe & Gerd.	A
G. claroides Schenck & Smith	H
G. clarum Nicolson & Schenck	A, H
G. constrictum Trappe	A, F–H
G. convolutum Gerd. & Trappe	H
G. corymbiforme Balszkowski	B
G. deserticola Trappe, Blows & Menge	E, H
G. dimorphicum Boyetchko & Tewari	H
G. etunicatum Becker & Gerd.	A, F
G. fasciculatum (Thaxter) Gerd. & Trappe	A, B, D, H
G. fecundisporum Schenck & Smith	A, H
G. fistulosum Skou & Jakobsen	H
G. gibbosum Balszkowski	B
G. globiferum Koske & Walker	A, E, H
G. halon Rose & Trappe	B
G. intraradices Schenck & Smith	G, H
G. lacteum Rose & Trappe	H
G. macrocarpum Tulasne & Tulsane	I

Table 2, Contd..

G. microaggregatum Koske, Gemma & Olexia	A,G, H
G microcarpum Tulasne & Tulasne	A, H
G minutum Balszkowski, Tadych & Medaj	B
G. monosporum Gerd. & Trappe	A, H
G. mosseae (Nicol. & Gerd.) Gerd & Trappe	B, H
G. nanolumen Koske & Gemma	G
G occultum Walker	A, H
G pallidum Hall	D
G. pansihalos Berch & Koske	A
G pubescens (Saccardo & Ellis) Trappe & Gerd.	A, H
G. pustulatum Koske, Friese, Walker & Dalpe	A, H
G. reticulatum Bhattacharjee & Mukerji	H
G. spurcum Walker	G
G. tortuosum Schenk & Smith	A, C, H
G. trimurales Koske & Halvorson	A
Sclerocystis pachycaulis Wu & Chen	H
S. rubiformis Gerd. & Trappe	A
S. sinuosa Gerd. & Bakshi	G

Spore density :

Mean or range of spore density of AM fungi in maritime sand dunes is given in Table 3. The spore density in Atlantic coast ranged from 0-3.4/g, while in Europe it was 0.05-0.95/g. Relatively the spore density in subtropical regions is higher than in temperate regions (0-11/g vs. 0-3.4/g). The spore density in tropical dunes ranged between 0 and 3.4/g.

Table 3.– Mean or range of spore density of arbuscular mycorrhizal fungi in coastal sand dunes.

Location	Spore density	Reference
Temperate locations		
Massachusetts, USA	0-0.7/g	85
Massachusetts, USA	0.24/g	128
Massachusetts, USA	0.32/ml (*Gigaspora gigantea)*	13
Rhode Island, USA	2.04	11
Rhode Island, USA	1.5-2.4/g	132
Rhode Island, USA	0.12-0.46 (*Gigaspora gigantea)*	39
Rhode Island, USA	1.92-3.36/g	47
New Jersey-Virginia, USA	0.64/ml	14
Alberese, Follonica and Migliarino,. Italy	0.95/g (*Glomus* sp.)	16
Tuscany, Italy	0.05-0.95/g	17
Tentsmuir, Scotland	0.06-0.62/ml	34
Subtropical locations		
New South Wales, Australia	1-11/g	18
Hasaki, Japan	2.87/g	41
Shirako, Japan	6.56/g	41
Florida, USA	0-6.77/g	36
Florida, USA	0.42-2.4/g	60
Baja California, Mexico	0-8/g	20
Santa Catarina, Brazil	1.7-3.8/g	9

Table 3, Contd..

Pakistan	0.01-0.3/g	25
Tropical locations		
Singapore	0.15/g	35
Kaua'i, Hawaii	0.09/ml	29
Mangalore, India	0.75/g	31
Mangalore, India	0-3.4/g	32
Mangalore, India	0-2.1/g	33
West coast, India	0.05-0.44/g	34

New species :

About 29 species of AM fungi have been described from the maritime sand dunes (Table 1). Out of this, 25 species have been described from temperate locations alone, among the rest, two each have been described from subtropical and tropical locations. This clearly indicates the lack of intense studies on AM fungi in tropical and subtropical san dunes.

Seasonal Periodicity

It is known that AM fungi sporulate differentially with change in time and host plant species[37]. The AM species richness and spore abundance was significantly correlated with the age of plants. The dune vegetation in fixed coastal sand dunes of Scotland revealed two peaks of root colonization during July and October-November[38]. A six-months seasonal study of AM fungal colonization of seven plant species in fixed coastal sand dunes of Baja California revealed up to 80% colonization[20]. Arbuscules were more when the soil was moist and vesicles were more abundant in dry condition. Gemma and Koske[39] studied spore abundance of the dominant fungus *Gigaspora gigantea* in the dunes of Rhode Island. The spore abundance increased from July and reached a peak in December indicating that a majority of spores formed during late summer and fall. The abundance of healthy and dead spores was reported to be opposing in the dunes of Rhode Island[15]. Seasonal dynamics of spores of five AM fungi in a sand dune of Massachusetts showed a bimodal trend of peak of spore abundance during May and October[13]. The spore density was highest during the month of summer (August) than in spring (May) and

fall (November) in the dunes of Florida[40]. Seasonal survey of AM fungi associated with *Spartina ciliata* in the dunes of Brazil did not show significant variations in spore abundance because of season[9]. But the spores of *Acaulospora* sp., *Glomus constrictum* and *G. etunicatum* were high in winter, while *Gigaspora albida* peaked during spring. No seasonal pattern was observed in AM spore number of three plant species in Japanese coastal sand dunes[41]. Several investigators observed the correspondence between the plant and fungal phenology[32,33,42-45]. Similar observations were made in the sand dune vegetation of Baja California[20].

Species Richness and Diversity

The AM fungi in most rhizosphere systems indicate the local species composition, while it has a strong historical component with ecological process[46]. In Australia the species richness was 1.5 in foredunes and it increased to 2.4 in the second dune ridge[18]. The mean species richness for the entire transect of New Jersey to Virginia was 4.9 (4.2-6.3) (ranged from 2-8 per root zone for *Ammophila* and *Solidago,* while for *Uniola* it was 6-14)[14]. The mean species richness was positively correlated with distance along transect and with temperature parameters. In Brazilian sand dunes the AM fungal richness index ranged from 4.6 (spring) to 6.8 (winter) with a mean of 5.9 for all samples[9]. The species richness of Brazilian sand dunes was similar to that of Atlantic coast of United States[14], but it was higher than that of the southern Australia[18] and Hawaiian Islands[28]. The AM fungal richness and diversity was consistently greater in moderately disturbed dunes than in severely disturbed dunes[34]. The spatial dispersion of AM species associated with the rhizosphere of American beach grass was analyzed at the depths of 10-18, 20-28 and 30-38 cm on the sand dunes of Rhode Island[47]. A highest of 3.36/g of AM spores was recovered at 20-28 cm depth. The spore abundance was significantly correlated to the depth of 20-28 cm and 30-38 cm than 10-18 cm. In Japanese coastal sand dunes, in rhizosphere of *Elymus mollis* AM spores were present up to a depth of 90-130 cm[41]. Maximum spores were recovered at 30-60 cm depth.

Sand Aggregation

Mycorrhizae are known to improve the soil structure and stability due to the formation of sand aggregates[48]. Three distinct phases of aggregate formation have been recognised : (i) AM hyphae entangle the soil particles; (ii) the roots and AM hyphae create conditions favourable to form microaggregates in soil; (iii) the roots and AM hyphae enmesh and bind the microaggregates to form larger macroaggregates[49]. The AM fungal hyphae entangles microaggregates and the polysaccharide

secretion firmly adheres the microaggregates. Up to 50 m of fungal hyphae form a network per gram stable microaggregate[50]. The AM fungal mycelia in sand dune soil stabilize the aggregates by the amorphous deposits of mycelia[51]. The AM fungi found in maritime sand aggregates along with bacteria, algae and other fungi[52]. A study of the association of *Glomus* with *Phaseolus vulgaris* showed that the weight of sand aggregates in non-mycorrhizal bean plants was 10g/kg, while it was 54g/kg in mycorrhizal plants[53]. The weight and the size of the aggregates increase from mobile to fixed sand dunes. The amount of aggregations usually increases with distance from the sea due to decreasing salinity, elevated nutrients and establishment of higher plant species. The AM fungal association with mycorrhizal plant in the maritime sand dunes stabilizes the dunes by binding the sand grains into wind resistant aggregates. The aggregates of sand dunes remain intact even after the death of roots and hyphae[10].

Association with Sand Dune Vegetation

In temperate locations most of the dune stabilizing plants belonging to the family Poaceae[54]. American beach grass is a major dune building plant species adapted to north-eastern Atlantic coast of United States[55] This plant species has the greatest influence on coastal geomorphology and plant community development[56]. Areas of the dunes with highest vegetation cover by American beach grass were associated with greatest AM spore density and diversity[11]. The species richness of AM fungi was nonlinear in the rhizosphere of American beach grass and *Solidago* along the latitudinal temperature gradient[14]. This phenomenon has been attributed to two reasons : (i) an increase in richness due to the effect of increased temperature from north to south; (ii) stimulatory effects of host plants due to warmer temperature towards south. The decline in species richness along further south was expected due to increased heat stress on the hosts. *Solidago* is also less frequent in southern Atlantic coast. Most favourable conditions (particularly temperature) are found at mid zones between New Jersey to Virginia for plant species as well as AM fungi.

Under coastal sand dune stabilization programme, *Ammophila* has been planted extensively along mid and north Atlantic coast of United States[57]. Artificial inoculation of AM fungi enhanced the transplanting success and establishment of *Ammophila*[56]. Planting *Ammophila* during October to April is ideal to stabilize the sand dunes of New England coast. This period corresponds to ideal temperature and moisture levels for survival of beach grass[58]. This period also corresponds to the peak of healthy inoculum of AM fungus, *Gigaspora gigantea*. Studies have been conducted on the pattern of AM fungal association of a large scale planting (age 0-7 years) of

Ammophila at Massachusetts. Over the successional sequence there was increase in the extent of AM fungal colonization of roots, community of AM spore population and the mycorrhizal inoculum potential of soil. The non-vegetated sites lack AM fungal propagules.

Ammophila arenaria (European beach grass or marram grass) is native to European coastal dune ecosystem. The AM fungal colonization was more extensive in the plant species established under European beach grass of British sand dunes[53]. The AM fungal colonization was increased from foredunes to fixed dunes in Scotland[59] Inoculation of *Glomus macrocarpum* and *G. fasciculatum* stimulated the growth of European beach grass grown in non-stabilized foreshore of Scotland[40]. South east Atlantic coast is naturally stabilized by perennial grasses particularly *Uniola paniculata* (sea oats)[60]. Association of sea oats with AM fungi reduces its environmental stresses and facilitates a rapid growth and dune stability. The established sea oats in Florida beach replenishment site showed elevated AM fungal root colonization, cfu of AM fungi and cfu of bacteria than in vacant and recently planted beach sites[60]. Performance of three AM isolates from Florida dunes (*Glomus deserticola, G. globiferum* and *G. etunicatum*) was tested on sea oats in green house trials[61]. Two isolates, *Glomus deserticoala* and *G. etunicatum* significantly increased the height, dry mass and plant cover of shoots of sea oats compared to controls. *Glomus globiferum* increased the shoot phosphorus content significantly, but did not affect the plant growth. Seedlings of sea oats inoculated with *G. deserticola* and *G. macrocarpum* at Miami beach, showed greater shoot dry mass (219%), root length (81%), plant height (64%) and number of tillers (53%) compared to the uninoculated plants[62]. Bacterial inoculation (*Klebsiella pneumoniae* or *Alcaligenes denitrificans* with *Glomus* spp. to sea oats enhances the AM root colonization[63]. Inoculation of *K. pneumoniae* increased the spore germination and hyphal growth of *G. deserticola.* Nitrogen fixing bacterial inoculation particularly *K.pneumoniae* enhances the AM symbiosis.

Most of the plant species established in the Gulf of Mexico (on the beach, embryo dunes, foredunes and stabilized dunes) were colonized by AM fungi[19]. In field bioassay, colonization of the seedlings of *Chamaecrista chamaecristoides* by AM fungi was more pronounced in the foredunes, but no mycorrhizal structures was noticed in mobile dunes[19]. Observation of seven dune plant species (from the beach, embryo dune, foredune and mobile dunes) suggested that the AM fungal colonization is crucial for the growth of early successional species[64]. The fixed coastal sand dunes of Brazil have a high AM fungal diversity and it has been related to the dominance of rhizomatous perennial grass, *Spartina ciliata*[9]. Members of the family *Asteraceae*,

Convolvulaceae, *Fabaceae* and *Poaceae* are dominant in tropical coastal sand dunes[31,65,66]. It was noticed that mycorrhizal indices were significantly higher in endemic plant species than in alien plant species at Hawaiin Islands[27]. High incidence of AM fungi has been found in the strand habitats of Galapagos Islands[67] and Heron Island, Great Barrier Reef[68]. High incidence of AM fungi in Hawaiin Island strand species agrees with the studies in Heron Island[68].

Spore Dormancy and Germination

The survival of AM fungal spores in sand dunes is dependent on biotic and abiotic factors. The spore dormancy facilitates overcoming the unfavourable conditions. The physicochemical features of coastal sand dunes influence the AM spore dormancy and germination. Different species of AM fungi may have different strategies of survival under wet and dry regimes of coastal sand dunes[69]. Spores of *Gigaspora* sp. lose their appearance, colour and viability when stored for period longer than 6-8 months due to ageing[70]. The dormancy of 2-9 weeks was seen in newly formed spores serving to maintain a high inoculum of AM fungi in soil during non-growing season of the host plant species *Ammophila* in Rhode Island[39]. The spores of *Gigaspora gigantea* gathered from the rhizosphere of *Ammophila* in Rhode Island, germinated on sand immediately after collection at 2% moisture and at temperature as low as 20 °C[70]. The spores of *G. gigantea* are not dormant in the absence of plant roots. Low temperature and low water potential prevent or delay the spore germination. Lack of such field condition in the sand dunes may not enforce spore dormancy. The AM fungi exhibits lower tolerance to higher carbon dioxide tensions and higher oxygen requirements for germination than other fungi[71]. The effects of sodium chloride on non-coastal AM fungal species showed a strong inhibition of germination to increasing salinity[72]. Coastal sand dune AM fungi are not continuously exposed to high salinites[72]. Germination of spores of *G. gigantea* was tested on immersion in seawater for 1-20 days[72]. Germination showed a significant linear decrease with the time of exposure, declined from 90% to 61% after 20 days. The germination was more reduced in the presence of chloride ions than sodium ions[73]. The recovery of germination of AM spores was more rapid if the spores were periodically exposed to sodium ions rather than chloride ions[27].

Fresh roots of *Sporobolus* immersed in seawater for seven days served as AM fungal inocula in pot culture. Thus, AM fungi are capable of oceanic dispersal through the leaf sheath of litter and rhizome segments[27]. Although a small amount of phosphorus in soil or agar improves the spore germination of AM spores, addition of KH_2PO_4 inhibits the germination of *Glomus mosseae*[74]. It is suggested that a

communication mediated by volatile messengers between plant and AM fungi results in directional growth of AM spore germ tubes towards roots and roots towards AM spores[75,76]. The colour of spores of *G. gigantea* changed from greenish-yellow to yellow to reddish-orange-brown to black (dead) due to attack of microbial parasites[15,77]. The first two kinds readily germinated, while latter two stages did not germinate[71].

Spore Ageing, Parasitism and Death

Ageing and death of AM spores on sand dunes are the common feature[15,47,77]. Newly formed healthy spores in temperate dunes undergo ageing and death within a period of about seven months due to parasitism by microorganisms[15]. Climatic and other conditions affect both the onset of sporulation and rate of spore ageing. Variation in the longevity of healthy spores may be dependent on edaphic factors. Based on the studies in Rhode Island dunes, a series of stages of spore vigour of *G. gigantea* was recognized by Lee and Koske[15] : healthy greenish-yellow, yellow with brown spots (mottled), reddish-orange-brown and collapsed/blackened/dead. Many species of Chytridiomycetes and Deuteromycetes have been identified as hyperparasites of spores of AM fungi[71]. Parasitism by fungi and actinomycetes resulted in decline of AM spore abundance and reduced the inoculum potential particularly during spring and summer in Rhode Island. Parasitized spores were found throughout the year in Rhode Island dunes[15]. Volatile products of soil actinomycetes reduced the germination of *Gigaspora margarita*[71]. Forty four species of fungi and six actinomycetes have been recovered from the surface-sterilized, crushed spores of *G. gigantea*[15]. Greater number of pathogens/saprophytes was isolated from older spores (brown and dead) than from newly formed spores. Predation and degradation of AM fungal spores in some tropical sand dunes have been reported to be greater than in temperate dunes[28]. The spore abundance in Hawaiian sand dunes was reduced due to microbial parasitism and predation which may favour the selection of non-sporulating AM species[28,29].

Dead spores of about 10 species of AM fungi of the genus *Gigaspora* have been found to be occupied by spores of other AM fungi (*Acaulospora, Gigaspora* and *Glomus*) in the sand dunes of Atlantic coast and Great Lakes of United States[78]. Spores of 1-5 different species occurred within a dead spore. The samples of Virginia beach contained the greatest percentage of occupied spores (individual spores contained 1-100 spores). In Hawaiian dunes, *Glomus microaggregatum* occurred inside the spores of *Scutellospora hawaiiensis*[29]. These spores would be overlooked in most collections unless larger, dead spores of *Scutellospora* are crushed and

examined[29]. *Scutellospora hawaiiensis* was also found inside the dead spores of *Gigaspora* sp.[79]. Association of one kind of AM spores with other kind of AM spores are of immense significance in terms of survival, dispersal and inoculum potential in coastal and dunes.

Effect of Edaphic Factors

Coastal sand dunes are deficient in nitrogen, phosphorus, potassium, organic matter and water[3]. Establishment of AM fungi in coastal sand dunes may be dependent on organic matter. Preferential association of AM fungi was found with particles of decaying organic matter[12,18,38,80]. Organic particles in the dunes serve as energy source[80]. Increased hyphal growth of AM fungi in soil with increased organic matter was evident[81]. The alkaline phosphatase activity by AM fungal mycelia was decreased in soil devoid of organic matter. Organic matter was expected to provide phosphorus as substrate for acid and alkaline phosphatase. Many studies in temperate locations have revealed that in sand dune ecosystem calcium and magnesium are found in adequate amounts for plant growth, while nitrogen, phosphorus and potassium are limiting[82]. Although the activities of AM fungal species are known with respect to the soil factors (heavy metals, texture, moisture, temperature, nutrient levels and salinity), the knowledge on the significance of these factors on the AM fungal diversity in natural ecosystems is inadequate[83]. There was little correlation between the density/frequency of occurrence of AM fungal population in the soil and the physicochemical characteristics (chloride, nitrate, phosphorus, potassium, organic matter, moisture and pH) with the exception of sand grain size in Rhode Island dunes[11]. There was no significant correlation between AM spore abundance and soil nutrients or edaphic features[13,14]. A significant difference was found in moisture, pH, phosphate, sodium, potassium and nitrogen between naturally vegetated and non vegetated dunes of the west cost of India which may be due to the effects of dune vegetation and AM fungi[31-34]. Among the three types of soil employed to grow the pigeon pea, the AM fungal colonization and their spore load were highest in coastal sand dune soil[84]. It was related to low levels of available phosphorus in sandy soil.

Impact of Dune Disturbance

Coastal sand dunes are susceptible for a variety of disturbances like tidal action, wind blow, erosion and sand accretion. Sea erosion is a major concern in temperate and tropical regions of the world. Extensive projects have been implemented to avoid beach erosion in Atlantic coast of United States[54,60,62,85]. In Atlantic coast besides replenishment of sand from offshore, beach grasses have been planted along the

beaches to initiate dune-building processes and thus to reduce the erosion losses. In south west coast of India, the beach areas prone to severe sea erosion are in the vicinity of coast-parallel river bends and associated coastal faults[86]. The artificial granite seawall shifts the erosion laterally and thus they become ineffective[87].

Seedlings exhibits stimulation of growth, tillering, reproductive output and biomass to partial burial[3]. This may possibly be due to intermediate disturbance of sand dunes. The intermediate disturbance facilitates the insulation of seedling root system from high sand temperature and desiccation, escape predation from surface feeders, greater access to soil moisture, more space for root, higher nutrient in the new substrate and change in the biota resulting in an increased plant species richness and diversity[3,34,88]. Burial of seeds, seedlings and plants in coastal sand dunes act as a strong selective force eliminates or reduces the less tolerant species and increases the abundance of tolerant species[89]. Similar criteria are applicable to the AM fungal spores to establish on the sand dunes. Observation on the burial of six tropical sand dune plant species showed an increased plant vigour (biomass and leaf area) and allocation of more biomass towards the aerial parts[90]. Possibly the AM fungal colonization as well as burial enhances the aerial biomass. Burial also influences the succession pattern of coastal vegetation and subsequently stabilizes the dune. Burial of *Ammophila* significantly stimulated the foliar growth rate and total plant dry mass; burial with AM fungal colonization irrespective of the presence or absence of plant parasitic nematodes elevates the foliar growth rate and total plant dry mass[91]. Burial of *Ammophila* also elevated the root dry mass, further elevation of root dry weight was found when plants with AM fungi was buried. When plants with AM fungi and nematodes were buried, the root dry weight was not drastically reduced, which indicate the inhibitory effects of AM fungi on nematodes.

The coastal reclamation in Singapore resulted in the establishment of non-mycorrhizal plant species due to lack of AM fungal inoculum[35]. Severe disturbance of sand dunes reduces or destroys the AM fungal propagules which results in the reduction or elimination of plant species[92]. Such severely disturbed areas require long period for re-establishment of mycorrhizal vegetation. These situations have major impact on natural plant succession on the dunes and their stabilization.

Impact of non-AM Endophytic Fungi

The fungal endophytic association provides a competitive advantage to the coastal sand dune vegetation against herbivores and other pathogens. A few studies have been conducted to understand the interaction between mycorrhiza and other plant endophytes[93]. Non-mycorrhizal endophytes are capable of altering the plant-plant

competition and drought resistance similar to that of mycorrhizae[94,95]. The secondary metabolites of non-AM fungal endophytes reduce the insect herbivory[95]. It is known that mycorrhizae reduce the *Acremonium*-induced insect herbivory[96]. Endophytic *Acremonium* sp. has reduced the colonization and reproduction by *Glomus macrocarpum* and *Glomus mosseae* in pearl millet[97,98]. The presence or absence of *Acremonium* can thus be employed to control the mycorrhizal community for the optimum effect on crop productivity[97]. In view of the effects of non-AM fungal endophytes on AM fungi and benefits to the dune vegetation, non-AM fungi of coastal sand dunes deserve special attention.

Succession of Higher Plants in Coastal Sand Dunes

The stabilization of disturbed ecosystems particularly coastal sand dunes is dependent on the successful establishment of the most effective plant community, which is possible by the association of plant species and AM fungi[35]. The dominance of non-mycorrhizal plants during the early succession has been attributed to the reduction or lack of mycorrhizal propagules after disturbance. Similar pattern of plant succession is applicable for the devastated or disturbed locations due to volcanic eruption[99] and coastal land reclamation[35]. A survey of vegetation on the reclaimed coastal land ecosystem in Singapore after five years indicated the absence of AM fungi and the dominance of non-mycorrhizal plant species. The plant cover was reduced from>95% to <10%, while the mycorrhizal plant species was reduced from >80% to 0% towards the sea. The zone close to sea was exclusively colonized by plant species belonging to non-mycorrhizal family, Cyperaceae. Obligate mycotrophic plant species are prevented from colonizing a site if the AM fungi are absent or their abundance is below the critical level[85]. But the facultatively mycotrophic non-mycotrophic plant species will be excluded when AM fungal inoculum potential is greater than the minimum level[100,101]. Francis and Read[101] studied the relative growth rate, survival of plant species belonging to the so-called non-host families (Brassicaceae, Chenopodiaceae and Polygonaceae) for 63 days in the presence and absence of AM fungal mycelia. These plant species showed a strong inhibition in their growth in the presence of AM mycelia, and some even suffered severe mortality. Absence of appresorium and arbuscules during the interaction between AM fungi and plant tissues suggest the lack of recognition event leading to the establishment of a functional symbiosis, instead it becomes a rather parasitic type of colonization[102]. Beyond the impact of AM fungi on plant growth and community structure, the role of AM fungi in coastal sand dune ecosystem dynamics has not been well understood[103].

Although the members of the family Caryophyllaceae colonize the disturbed habitats, *Silene colorata* and *S. orites* were found to be colonized in stable sand dunes without much human interference on the west coast of Italy than on the eroded and disturbed dunes by human activities[16]. The frequency of occurrence (60-100%) and the mycorrhizal index (1.5-3) and root colonization of AM fungi increased with the age of the substrate (8-137 years)[26]. Similar observations were made at the volcanic sites of Mount St. Helen's mountains, United States[104] and Hawaiian Islands[26]. Non-mycorrhizal plant species do not support AM fungal hyphae but may encourage the proliferation of other non-symbiotic fungal mycelia through the release of carbon compounds in the vicinity of the roots[105]. Hence, the association and role of non-mycorrhizal fungi on sand dune plants needs more attention. The soil phosphorus was generally low in coastal sand dunes, but was significantly high at the high tide line where non-mycotrophic annuals establish including the reclaimed area[106]. The plant succession initiation by non-mycotrophic or facultative mycotrophic species depends on the degree of disturbance and the soil fertility. Biomes with high soil fertility are usually colonized by high proportion of non-mycotrophic plant species, as in arid and semiarid lands. Succession in biomes with low fertility is initiated primarily by facultative mycorrhizal plant species, as in tropical forests or in sand dunes[106,107]. In coastal sand dunes the extent of disturbance determines the pattern of vegetation. If the disturbance eliminates AM fungi, non-mycorrhizal plants get established, in highly disturbed conditions (erosion/accretion) all plant species may be considerably eliminated[91]. Thus, based on the extent of disturbance, strategies have to be developed to inoculate the AM fungi and suitable vegetation on coastal sand dunes for restoration and stabilization.

Future Outlook

Below ground diversity of AM fungi is one of the major factors governing the maintenance of plant diversity and the ecosystem functioning[108]. The loss of AM fungal diversity decreases both plant equilibrium and ecosystem productivity, which leads to the instability of ecosystem[100]. Although a few studies have been conducted on the AM fungi of tropical coastal sand dunes, the species richness is higher than temperate and subtropical locations. A wide variety of plant species (sedges, shrubs, herbs, climbers, creepers and tree species) which have been adapted to tropical coastal sand dunes[27,30,31,64,66,109] offers excellent scope for re-vegetation, restoration and stabilization of coastal dunes. The mat-forming creepers (*Ipomoea pes-caprae* and *Launaea sarmentosa*) including nitrogen fixers (*Alysicarpus rugosus, Canavalia cathartica, C. maritima*), rhizomatous members of Cyperaceae and *Spinifex littoreus*

would be the ideal plant species in the restoration and stabilization of tropical coastal sand dune ecosystem. Multistoried cropping pattern on dunes would facilitate to withhold the sand movement and withstand the sand accretion. The AM fungal species which are abundant and have a wide host range (*Gigaspora margarita, Glomus albidum, G. lacteum, Scutellospora erythropa* and *S. gregaria*); high frequency of occurrence and wide geographical distribution (*Gigaspora gigantea, Glomus mosseae* and *G. dimorphicum*) would facilitate the maintenance of plant-AM fungal mutualism, enrich and restore the coastal sand dune ecosystem. Coastal sand dunes being extreme habitats provide excellent opportunities to understand the mutual interaction and adaptation of biota to environmental stress. The coastal sand dune stress-tolerant plant species and microbes deserve special attention for their application in agriculture, horticulture, forestry, environmental rehabilitation and bioremediation.

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Development of near isogenic lines of productive silkworm breeds by isozyme marker based selection

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Abstract

Of late, molecular marker facilitated investigations using isozyme/DNA markers is being widely explored in the breeding of crop plants and live stock for the improvement of desirable traits. In India, limited success of conventional breeding and selection strategies adopted so far in silkworm breeding, has warranted testing of the new strategies for maximising yield realisation. In this direction, work carried out earlier on a number of biochemical parameters has shown the prospects of using digestive amylase as a marker in silkworm breeding due to its close association with survival, better digestibility and isozyme polymorphism. A test breeding plan was adopted using the indigenous low yielding polyvoltine breeds, namely, Pure Mysore and Nistari having '4 band' and '5 band' cathodic amylase isozyme types, respectively, as donor parents (DP) and the productive bivoltine breeds like NB18, CSR2 and CSR5 with 'null' type of isozymes as recurrent parent (RP). Near Isogenic Lines (NILs) of the RPs were developed by introgressing the isozymes from the DPs via backcross breeding strategy. The evolved lines have shown significant improvement in viability over their respective RPs without any deterioration in yield traits. The laboratory evaluation has indicated superiority of a few hybrid combinations of the evolved lines over the conventional hybrids in terms of higher index values for survival and other economic traits. The methodology of the breeding scheme, the performance of the evolved lines and the future plan are discussed.

(**Key words :** amylase isozyme/backcross breeding/Near Isogenic Lines/silkworm)

Introduction

Sustained breeding efforts by conventional methods since the last three decades has resulted in the evolution of a number of productive Indian silkworm breeds[1,2]. These have played a pivotal role in augmenting silk production resulting in over ten fold increase from the '60s to the '90s. Inspite of achieving a quantum jump in production, the twin problems of low productivity and poor fibre quality continue to impair the vertical growth of silk industry.

To overcome these drawbacks, new breeding strategies adopted during the early '90s have resulted in the evolution of improved polyvoltine breeds like RD1, P2D1, BL lines[3] as well as productive bivoltine breeds of CSR series with high shell ratio and raw silk recovery[4] which have the potential of producing 4A international grade silk. However, the full potential of these evolved breeds has not been realised as evident from the large yield gap between the laboratory results and farmers' field which is indicated by the on-farm trials of these breeds conducted in the recent past. The possible reasons for the limited success of these breeds in the field could be the cumulative result of the interplay of poor survival potential of the new breeds, the low input-cum-low management situation prevalent at most of the farmers' places and the vagaries of the tropical environment.

Rapid advances in genetics and molecular biology have opened up new avenues for overcoming the limitations of traditional breeding methods for improving the efficiency of selection of the target trait, maximise the rate of improvement and shorten the breeding time. With regard to silkworm breeding in India, the present situation warrants testing of the novel breeding strategies which have been offered by the recent developments in molecular biology and biotechnology, for improving yield and productivity. Of late, one of these new techniques successfully being used in plant and animal breeding is marker assisted selection (MAS) employing isozyme or DNA markers for the transfer of desirable traits from phenotypically inferior parents into productive breeds.

Prospects of molecular marker assisted backcross breeding

In recent years, molecular marker facilitated backcross breeding using isozyme/DNA markers are being widely explored for introducing desirable traits in crop plants[5-8] and live stock[9-13].

Recurrent backcross breeding has been the method of choice for gene introduction when the recipient line (recurrent parent) has many desirable properties but lacks a specific trait that is known to be present in a donor parent. The introgression of the desired gene from the donor consists of raising the F1 between the donor and the recurrent parents. The F1 is then backcrossed to the recurrent parent and the progeny is screened with respect to the character expressed by the target gene being introduced. The process is repeated by raising several backcrosses and the population progressively becomes more like a recurrent parent.

Theoretical basis of backcross breeding

As discussed in detail by Allard[14] and Welsh[15], the genetic background can be considered in terms of the total information contributed by either parent. In the F1, the genetic information from each Parent is 50%. As we begin to backcross, contribution from the donor parent (DP) is gradually increased by 50% with each backcross and is calculated by the formula $(1/2)^n$ where 'n' is the number of backcrosses. At BC1, 75% of the genetic background would be of the recurrent parent (RP), while that of the DP would be 25%. Further, at BC2, it would be 87.5% and 12.5% respectively. Finally at BC10, the RP recovery would be about 99.95%, while the contribution of the DP is reduced to 0.05% (Fig. 1)

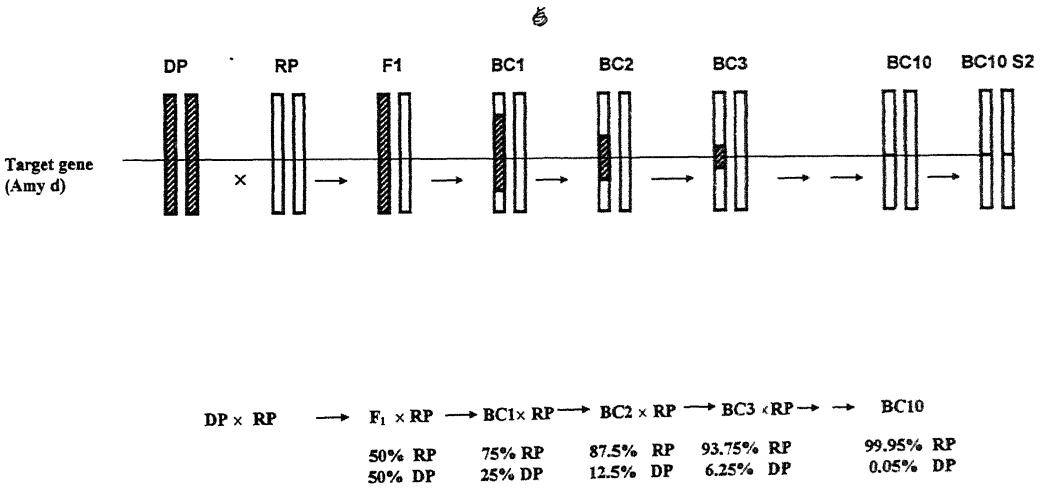

Fig. 1–Theoretical basis of backcross breeding showing the introgression of the target gene (Amy d) from the donor parent (DP) into the genetic background of the recurrent parent (RP). Please see text for details.

Further, in the F1, all the loci differing between the parents will be heterozygous. With backcrossing, increased homozygosity for the recurrent parent will occur. The rate at which this takes place is according to the formula : (2m-1)/2m, where 'm' is the

number of backcrosses. For example, after the 10^{th} backcross, 99.9% of the segregating loci will be homozygous. Besides, if only one loci is selected from the donor parent, the proportion of genotypes homozygous would also be 99.9% as obtained by the formula $\{(2m-1)/2m\}^n$, where 'n' equals the number of heterozygous loci in the original F1 and 'm' equals the number of backcrosses.

If the parents used are quite similar genetically, minimal number of backcrosses may be sufficient for the RP recovery. In the case of genetically divergent parents, several backcrosses may be required to eliminate the undesirable genetic background of the DP. Further, linkage is another factor which affects introgression. If the target gene is linked with other undesirable genes, reduced speed of RP genotype recovery will occur. During the process of backcrossing, if the selected trait is controlled by a dominant gene, there is very little problem as selection of the progeny can be performed directly. In case of traits governed by recessive genes, alternate selfing operation has to be carried out subsequent to every backcross, to recover the homozygous recessive progeny.

After raising the required number of backcrosses, a line is obtained (that carries the target gene of the donor parent in the background) which is nearly identical to that of the recurrent parent. This line is designated as Near Isogenic Line (NIL). Most of the currently available NILs in plants have been developed by using 5-6 backcrosses. Thus a number of NILs have been generated in various crop plants as the by-products of introgression of the target gene by backcross breeding. These NILs are being successfully used to identify DNA markers that are closely linked to traits of interest[16].

Identification of enzyme marker in mulberry silkworm for backcross breeding

In silkworm, earlier investigations on a number of biochemical parameters across a wide array of germplasm stocks have under scored the importance of amylase because of its significant relationship with survival and yield traits[17].

Further, screening of germplasm stocks for the analysis of cathodic digestive amylase by PAGE revealed three types of isozyme polymorphisms (Fig 2). It is reported that the cathodic amylase is controlled by a single gene, 'Amy d' of the 8^{th} linkage group (locus 2.8 cM) with three multiple alleles, i.e., Amy d^v, Amy d^{iv} and Amy d^n representing '5 band', '4 band', and 'null' pattern[18]. Allthe productive bivol-

tines were of the 'null' type with very low activity, while all the polyvoltine high survival stocks were either of the '4 band' or '5 band' types having many fold high activity[19].

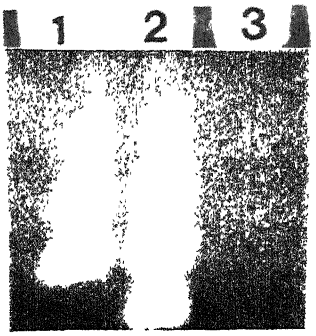

Fig 2–Digestive amylase isozyme polymorphism in silkworm stocks as revealed by cathodic PAGE. Lane 1 : '4 band' type found in Pure Mysore. Lane 2: '5 band' type of Nistari Lane 3 'null' type as shown in NB18, CSR2 and CSR5 where no bands were seen

In view of the wide genetic divergence in terms of activity and isozyme polymorphism and also because of its role in better digestibility and close association with survival, digestive amylase isozyme has been chosen as a surrogate marker in silkworm breeding for improving survival potential of the productive breeds.

Selection of parents

Indigenous polyvoltine silkworm breeds, namely, Pure Mysore with '4 band' and Nistari with '5 band' cathodic amylase types were chosen as the Donor Parents (DP). These two breeds are being reared in India for more than hundred years, the former being popular in south India and the latter in north India. The survival potential of these breeds are high which are being reared through out the year but the yield and fibre quality is poor.

Three productive bivoltine breeds, viz., NB 18, CSR2 and CSR5 with 'null type of amylase were selected as Recurrent Parents (RP). NB18 was commercialised in the early '70s but due to the low survival potential in the tropical climate, the yield realised in the field was poor and hence it is primarily being used as a male component in combination with the local polyvoltine breeds for cross breed preparation. CSR2 and CSR5 were recently authorised in 1997 for commercial use, which are highly productive with high shell ratio and superior yarn quality. These

breeds require good quality leaves and better management practices during rearing and hence presently they are being tested with selected progressive farmers during favourable seasons only. Thus, these three productive breeds were selected RPs in the present breeding scheme for improving their survival potential by using the indigenous breeds as DPs employing isozyme marker assisted selection.

The breeding scheme

A backcross breeding scheme was adopted using the Pure Mysore and Nistari which were homozygous for '4 band '(4/4) and ' 5 band '(5/5) amylase isozyme types respectively as donor parents (DP) and three productive bivoltine breeds, namely, NB18, CSR2 and CSR5, all with 'null' (N/N) type as recurrent parents (RP).

F1 progeny were raised between the DPs and the RPs. The F1 females which were heterozygous for the isozyme pattern (4/N or 5/N) were backcrossed to the RP (Fig 3). The BC1 progeny were again backcrossed to the RPs. In BC2, two types of beds were expected. In one type, 50% of the progeny heterozygous (4/N or 5/N) and the other 50% homozygous for the 'null' (N/N) pattern. In the other type of bed, all the progeny (100%) to be homozygous for the null (N/N) pattern. In order to select the individuals having the donor isozyme pattern, the BC2 progeny were reared in 30 cellular beds and the progeny were electrophoretically screened bedwise and the progeny showing the heterozygous (4/N or 5/N) pattern were selected and backcrossed again to the RP. The progeny showing homozygous pattern for the null (N/N) were culled.

The procedure was repeated and ten backcrosses were made with the RP. At BC10, the progeny were still in the heterozygous condition for the amylase isozymes, i.e., 4/N or 5/N and hence they were selfed to obtained the homozygous pattern (4/4 or 5/5) of the donor isozymes. Hence, the BC10 progeny were subjected for individual screening for the isozyme and the progeny (4/N or 5/N) were selected and BC10 S1 generation was raised. In the BC10 S1 bed, 25% of the progeny were homozygous (4/4 or 5/5) and 50% will be heterozygous and the remaining 25% homozygous for the 'null' (N/N) type.

During electrophoresis, the homozygous (4/4 or 5/5) could not be distinguished from the heterozygous (4/N or 5/N) pattern in the gel. Hence, in order to select the homozygous progeny, a second selfing was required and followed by a test cross with N/N for distinguishing homozygotes from heterozygotes.

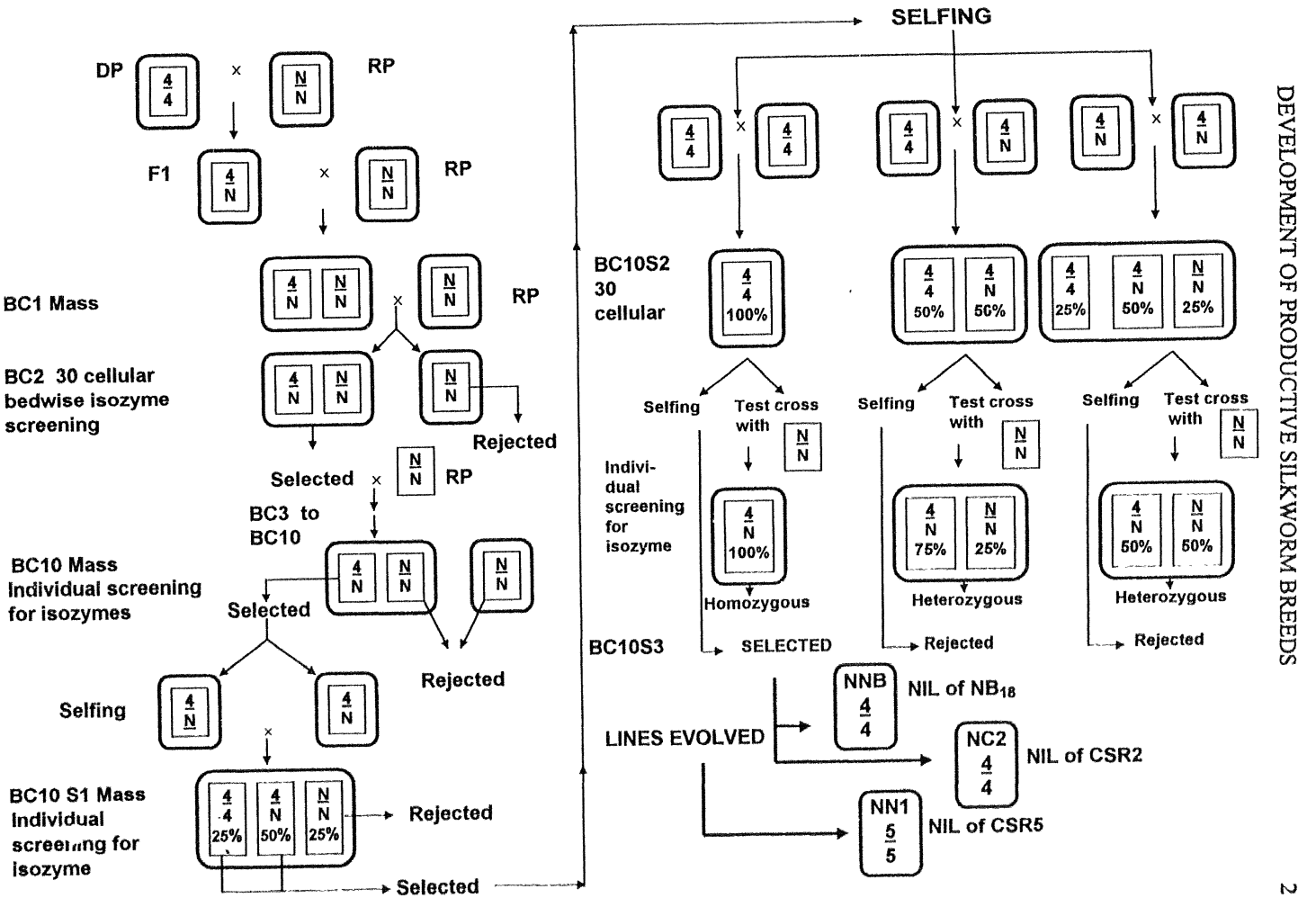

Fig. 3.–The selection scheme for introgressing '4 band (4/4), amylase isozymes from donor parent (DP) into the genetic background of 'null(N/N)' type recurrent parent (RP) leading to the development of Near Isogenic Lines (NILs' Please refer text for further details.

So, the BC10 S1 progeny were subjected for enzyme analysis individually and the progeny showing the 4 band or 5 band phenotypes were selfed and BC10 S2 families were raised and reared as 30 cellular beds. In each bed 10 batches of BC10 S3 progeny were raised by random mating and the layings were hibernated. Further, test cross progeny of BC10 S2 x RP were also raised in each bed which were reared cellularly. Each bed of the test cross progeny were again individually screened enzymatically and the progeny having all individuals of 4 and 5 band phenotypes were identified and the corresponding BC10 S3 progeny with the homozygous (4/4 or 5/5) pattern of the donor isozymes were selected and retained.

Thus, three Near Isogenic Lines, namely, NNB (NIL of NB18) and NC2 (NIL of CSR2) with 4 band pattern and NN1 (NIL of CRS5) with the 5 band pattern were derived by introgressing the isozymes from the multivoltine donors i.e., Pre Mysore and Nistari respectively.

Performance of the evolved lines

The rearing performance of backcross generations NNB, NC2 and NNI lines from BC1 to BC9 as well as the selfed generations from BC3 to BC10 vis-a-vis their respective recurrent parents under seventeen crops between April 1995 and June 99 period is depicted in Table 1. In these generations, only few progeny were sampled showing the isozyme type of the DP and the entire beds were selected and continued and the rearing data are available. While, the selfed generations at BC10, BC10 S1, BC10 S2, which was followed by a testcross, only about 100 female and male larvae each were subjected for individual screening for isozyme type of the DP and selfed, hence, the rearing data are not available. Perusal of the data of NILs reveal that the evolved lines have consistently performed well in all the generations in comparison to their respective RPs. The evolved lines have also shown improvement in survival (represented as Yield/10000 larvae by number), at the same time maintaining the cocoon characteristics on par with those their recurrent parents.

The mean estimates of the rearing data of the evolved lines and their respective RPs were subjected for 't' test which revealed significant increase in survival in the evolved NILs over RPs (Table 2). In case of cocoon traits, though marginal decrease was noticed in the NILs, the differences were not significant except for shell ratio in NC2.

Table 1.– Comparative rearing performance of NILs vis-a-vis RPs.

Gen.	Season	Yield/10000 larvae By No.	By wt. (Kg)	CWT (g)	SWT (g)	SR (%)	Gen.	Yield/10000 larvae By No.	By wt. (Kg)	CWT (g)	SWT (g)	SR (%)
NIL : NNB							**RP: NB18**					
BC1	Apr.95	9599	15.6	1.760	0.331	18.8	1	9320	14.8	1.656	0.363	21.9
BC2	Jun.95	8391	12.3	1.540	0.290	18.8	2	7447	8.8	1.420	0.280	19.7
BC3	Aug.95	8290	13.7	1.780	0.347	19.5	3	7866	11.6	1.600	0.309	19.3
BC4	Oct.95	9196	14.3	1.629	0.315	19.3	4	8640	13.9	1.740	0.340	19.5
BC5	Dec.95	8655	11.6	1.390	0.260	18.7	5	8567	14.8	1.480	0.274	18.5
BC6	Feb.96	8785	11.4	1.355	0.263	19.4	6	7408	11.3	1.522	0.301	19.8
BC7	Apr.96	8280	14.3	1.422	0.281	19.8	7	8083	9.8	1.289	0.236	18.3
BC8	Jun.96	8360	11.9	1.565	0.314	20.1	8	7540	10.8	1.547	0.314	20.3
BC9	Aug.96	9400	14.7	1.571	0.301	19.2	9	8653	13.5	1.768	0.322	19.2
BC10 S3	Sep.97	9086	13.6	1.518	0.291	19.2	10	8266	13.0	1.612	0.316	19.6
BC10 S4	Nov.97	8593	13.9	1.697	0.338	19.9	11	8067	14.6	1.886	0.362	19.2
BC10 S5	Jan.98	9100	14.3	1.531	0.318	20.8	12	8664	15.6	1.707	0.350	20.5
BC10 S6	Mar.98	8552	12.1	1.407	0.288	20.5	13	8316	13.1	1.578	0.320	20.3
BC10 S7	Jun.98	7293	10.2	1.362	0.258	18.9	14	7120	9.23	1.293	0.267	20.6
BC10 S8	Aug.98	8814	12.9	1.481	0.290	19.6	15	7568	12.1	1.583	0.297	18.8
BC10 S9	Oct.98	8547	13.3	1.440	0.264	18.3	16	7720	12.2	1.567	0.289	18.4
BC10 S10	Dec.98	9413	17.1	1.795	0.374	20.8	17	9000	16.0	1.824	0.364	20.0
Mean		8727	13.4	1.544	0.301	19.5		8132	12.6	1.587	0.312	19.6
SD		540	1.7	0.141	0.032	0.7		602	2.1	0.157	0.035	0.9
NIL : NC2							**RP: CSR2**					
BC1	Jun.95	9000	14.4	1.580	0.330	21.5	1	6080	8.3	1.390	0.322	23.2
BC2	Aug.95	8877	16.0	1.620	0.362	21.0	2	8288	15.3	1.910	0.397	20.8
BC3	Oct.95	8440	14.8	1.762	0.372	21.1	3	7576	12.5	1.590	0.386	22.1
BC4	Dec.95	8530	13.0	1.580	0.329	20.8	4	8133	14.3	1.580	0.322	20.4
BC5	Feb.96	9164	13.2	1.453	0.309	21.3	5	8133	12.5	1.575	0.357	22.7
BC6	Apr.96	8944	17.4	1.333	0.286	21.5	6	8173	10.5	1.334	0.286	21.4
BC7	Jun.96	8968	14.3	1.598	0.336	21.0	7	8240	11.9	1.678	0.360	21.5
BC8	Aug.96	9170	15.7	1.743	0.377	21.6	8	8816	16.0	1.800	0.396	21.9
BC9	Oct.96	8980	15.9	1.830	0.405	22.1	9	8640	15.5	1.875	0.422	22.5
BC10 S3	Nov.97	8773	16.6	1.952	0.417	21.4	10	8347	17.5	1.961	0.447	22.8
BC10 S4	Jan.98	9192	14.8	1.629	0.353	21.7	11	8560	14.6	1.659	0.381	23.0
BC10 S5	Mar.98	8948	15.4	1.624	0.350	21.6	12	8568	14.9	1.649	0.385	23.3
BC10 S6	Jun.98	7894	10.7	1.379	0.287	20.8	13	6936	10.0	1.434	0.329	22.9
BC10 S7	Aug.98	8253	12.3	1.490	0.310	20.8	14	7776	11.7	1.557	0.340	21.8
BC10 S8	Oct.98	8717	14.3	1.819	0.387	21.3	15	8376	16.3	1.902	0.422	22.2
BC10 S9	Dec.98	9193	19.4	2.098	0.465	22.2	16	9088	19.7	2.182	0.516	23.6
BC10 S10	Feb.99	9547	16.3	1.707	0.374	21.9	17	9408	16.9	1.801	0.414	23.0
Mean		8858	15.0	1.670	0.358	21.4		8190	14.0	1.709	0.383	22.4
SD		388	2.0	0.196	0.016	0.4		762	2.9	0.216	0.055	1.0

Table 1–Continued....

Gen.	Season	Yield/10000 larvae By No.	By wt. (Kg)	CWT (g)	SWT (g)	SR (%)	Gen.	Yield/10000 larvae By No.	By wt. (Kg)	CWT (g)	SWT (g)	SR (%)
NIL : NN1							RP: CSR5					
BC1	Oct.95	9700	18.1	1.885	0.382	20.3	1	8512	15.8	1.872	0.407	21.7
BC2	Dec.95	9066	14.9	1.680	0.313	18.6	2	8480	14.4	1.680	0.331	19.7
BC3	Feb.96	8825	13.3	1.507	0.315	20.9	3	7184	11.8	1.652	0.355	21.5
BC4	Apr.96	8580	11.7	1.369	0.287	21.0	4	8040	10.2	1.354	0.286	21.1
BC5	Jun.96	9088	13.3	1.521	0.307	20.2	5	8651	14.0	1.633	0.353	21.6
BC6	Aug.96	9260	14.7	1.644	0.326	19.8	6	9500	16.8	1.744	0.354	20.3
BC7	Oct.96	8600	13.9	1.647	0.341	20.7	7	7925	13.5	1.794	0.372	20.7
BC8	Nov.97	9708	14.7	1.731	0.364	21.0	8	8373	14.0	1.699	0.340	20.4
BC9	Jan.98	9381	14.5	1.518	0.324	21.3	9	9320	15.5	1.603	0.330	20.6
BC10 S3	Mar.98	8452	12.9	1.543	0.343	22.2	10	8320	12.1	1.482	0.344	23.2
BC10 S4	Jun.98	7757	10.1	1.250	0.254	20.3	11	7168	9.4	1.305	0.285	21.8
BC10 S5	Aug.98	8154	11.2	1.473	0.310	21.0	12	7488	10.1	1.454	0.315	21.7
BC10 S6	Oct.98	7777	13.1	1.582	0.321	20.3	13	7555	12.2	1.576	0.318	20.2
BC10 S7	Dec.98	8907	17.2	1.918	0.423	22.1	14	8880	16.6	1.919	0.443	23.1
BC10 S8	Feb.99	9460	15.9	1.696	0.361	21.3	15	9016	14.4	1.563	0.347	22.2
BC10 S9	Apr.99	7680	9.7	1.313	0.282	21.5	16	7520	9.8	1.407	0.308	21.9
BC10 S10	Jun.99	8052	11.5	1.514	0.319	21.1	17	7560	11.6	1.521	0.329	21.6
Mean		8732	13.6	1.576	0.320	20.8		8207	13.1	1.602	0.342	21.4
SD		655	2.2	0.174	0.039	0.039		706	2.3	0.168	0.038	0.9

Gen = Generation; CWT = Cocoon weight; SWT = Shell weight; SR% = Shell ratio % (SWT x 100/CWT)

Table 2–Results of 't' test between the evolved NILs their respective RPs.

			< ----------t value---------- >				
NILs	vs	RPs	Yield (By No.)	Yield (By wt.)	Cocoon wt.	Shell wt.	Shell Ratio
NNB	vs	NB18	3.032**	1.112(NS)	–0.849(NS)	–0.914(NS)	–0.508(NS)
NC2	vs	CSR2	3.221**	1.108(NS)	–0.458(NS)	–1.496(ns)	–3.849***
NN1	vs	CSR5	2.250*	0.637(NS)	–0.439(NS)	–1.096(NS)	–1.922(NS)

*, ** and *** denote significance of 't' at 5%, 1% and 0.1% respectively, NS = Not significant.

For evaluation of these evolved NILs and also to identify promising hybrid combinations for further trials, eight hybrid combinations were prepared along with control hybrid KA x NB4D2 which is the traditional bivoltine commercial hybrid reared by the farmers since the last two decades. These hybrids were reared under six trials during 1998-99 period. From the rearing performance of these hybrids under six trials (Table 3), it is evident that all the eight hybrids have shown improvement over the control not only in survival (Yield/10000 larvae), but also on productivity traits like shell wt., shell ratio, filament length, renditta, raw silk % etc. Further, for identifying the potential hybrid combinations, they were evaluated by multiple trait index. For calculating the index values, the negative traits like total larval duration, V age larval duration, denier and renditta, where lesser values are desirable have been omitted and the remaining nine traits were included. Evaluation index (E.I.) was calculated[20] for each trait of each hybrid as per the following formula :

$$E.I. = \frac{A-B}{C} \times 10 + 50$$

Where, A = value obtained for a particular hybrid for particular trait
B = Mean value for a particular trait of all hybrids
C = Standard deviation of a particular trait for all hybrids.
10 = Standard unit
50 = Fixed value

Table 3.– Rearing performance of hybrids developed by isozyme selection (Mean of six trials – May '98 to April '99)

SL. NO.	HYBRID	Eggs/ DFL	Total L. D. (Hours)	V Age L. D (Hours)	Yield/10000 larvae By No.	By wt. (Kg)	Cocoon weight (g)	Shell weight (g)	Shell Ratio (%)	F. L. (m)	Den-ier	Ren-ditta	Raw Silk (%)	Ree-labi-lity
1	NC2 x NN1	484	492	136	9320	17.2	1.793	0.384	21.4	1037	2.40	6.50	16.0	82.0
2	NC2 x CSR5	462	496	140	9044	17.1	1.800	0.391	21.7	979	2.55	6.33	16.1	88.3
3	NC2 x NNB	462	490	139	9107	16.6	1.736	0.363	20.9	902	2.40	6.51	15.8	84.2
4	NC2 x NB18	473	485	136	9196	16.5	1.774	0.373	21.0	932	2.40	6.50	15.6	85.4
5	CSR2 x NN1	492	489	137	9458	17.3	1.806	0.404	22.4	1014	2.47	6.39	16.3	83.4
6	CSR2 x CSR5	499	496	138	9129	16.8	1.789	0.406	22.7	1099	2.49	6.05	16.9	80.2
7	CSR2 x NB18	504	489	133	9189	17.1	1.796	0.383	21.3	1011	2.58	6.44	15.9	83.0
8	CSR2 x NNB	522	488	135	9403	17.3	1.796	0.385	21.5	947	2.41	6.38	16.1	80.1
9	KA x NB4D2 (Control)	522	494	133	9062	16.7	1.841	0.360	19.5	966	2.23	7.23	14.4	81.4
Mean		491	484	136	9212	16.9	1.792	0.383	21.4	987	2.44	6.48	15.9	88.9
SD		22	20	2	141	0.3	0.026	0.015	0.9	57	0.10	0.29	0.6	1.6

DFL = Disease Free Laying L.D. = Larval Duration F.L. = Filament Length

Mean index values were calculated for the nine traits for the nine hybrid combinations reared under six trials. The index values for survival as well as the overall index values for nine traits (Table 4) indicate that for survival, except NC2 × CSR5, the remaining seven hybrid combinations have scored higher index values over the control hybrid, KA × NB4D2 and five hybrids have higher values than the hybrid of the Recurrent Parents, i.e., CSR2 × CSR5. Further, in terms of overall index values for nine traits, seven hybrids were found to be superior over the control. The hybrid NC2 × NN1, where both the parents have been introgressed with isozymes from the DPs, the hybrids, CSR2 × NN1 and CSR2 × NNB, where one of the parents were of the isozyme type of the DPs have scored higher overall index values. Among all the hybrids, CSR2 × NN1 has scored the highest index values for survival (67.5) as well as all the nine traits (58.6) exceeding the values of not only the control hybrid but also CSR2 × CSR5. It is interesting to note that in the hybrid CSR2 × NN1, CSR2 is of 'null' and NN1 is of the '5 band' type of amylase. This indicates the possibility of higher heterotic vigour of 'null' and high enzyme activity combinations than the others. Even though at the laboratory level, other than CSR2 × NN1, the remaining hybrids having parents introgressed with isozymes from the DPs have not outscored the values of CSR2 × CSR5, the differences could be more pronounced under the field situation.

Table 4–Mean Index values of the hybrids (average of six trials)

Sl. No.	Hybrids	Index Value for	
		Survival	All traits
1	NC2 x NN1	57.7	52.3
2	NC2 x CSR5	38.1	49.7
3	NC2 x NNB	42.5	40.8
4	NC2 x NB18	48.9	45.6
5	CSR2 x NN1	67.5	58.6
6	CSR2 x NB18	48.4	51.8
7	CSR2 x CSR5	44.1	54.9
8	CSR2 x NNB	63.6	52.7
9	KA x NB4D2 (Control)	39.3	43.6

It is expected that few of the new hybrids developed by isozyme selection may perform better than the existing hybrids in the field. Hence it has been planned to conduct multilocational trials of few of these hybrids selected on the basis of index values, at regional research stations during 1999-2000 for identifying the promising hybrid combinations for large scale field testing.

Concluding Remarks

Molecular marker assisted backcross breeding is being successfully used for introducing desirable traits from the donor to the recipient line and as the offshoot of this breeding process, a number of NILs have been developed in various crop plants and live stock. It has also been reported that phenotypically inferior wild varieties when crossed with the high yielding domesticated varieties have shown transgressive variation[21] which has been used to improve many traits of economic importance. Wild relatives have been exploited as a source of disease and insect resistance in may crops but their use for the transfer of this trait is less attractive during breeding as the transfer of the target trait is often accompanied by many linked undesirable characters. This phenomenon is referred to as 'Linkage drag[22]' Thus intensive selection has to be carried out to recover the productive lines. However, it has been shown that using molecular marker assisted selection, this linkage drag can be minimised[5,8].

In the case of silkworm also, earlier breeding programmes using the indigenous poor yielders like Pure Mysore and Nistari as one of the parents has resulted in limited improvement in the evolved lines when phenotypic selection alone was employed. In the present breeding scheme, eventhough low yielding parents have been used as donors, selection based on amylase isozymes has not resulted in any deterioration in the cocoon traits in the evolved lines, while there has been significant improvement in the survival potential.

Since survival is a complex trait, it is not possible to substantiate how selection of '4 band' or '5 band' amylase isozymes with high activity is directly responsible for high survival. However, earlier it has been shown that races with high amylase content could digest and survive better when poor quality leaves or artificial diet was fed when compared to races with low amylase content[23]. Further, low amylase activity stocks were found to excrete five fold more starch than the polyvoltine stocks with high amylase activity[24]. It means that the former cannot digest and assimilate starch adequately which may be affecting the carbohydrate metabolism, which in turn could result in lower fitness by some pathway which is not known presently and further in depth studies are needed to support this contention. It is believed that increased enzyme activity and efficient starch digestion may confer better adaptability under the

tropical condition here the leaf quality is generally poor and hence could result in better survival.

Gimelfard and Lande[25] have suggested the potential utility of combining molecular information with phenotypic selection for improving the efficiency of selection and maximising the rate of improvement of the selected trait. Thus the new strategy employed in the present study by integrating isozyme based selection with the conventional phenotypic selection has clearly shown the prospect of its use in silkworm breeding.

In the recent years there has been rapid developments in genome analysis of silkworm and the RAPD/RFLP maps have already been published[26,27]. Application of PCR based RAPD and also DNA finger printing with minisatellite probes have revealed distinct DNA profiles between diapausing and non-diapausing stocks[28,29]. It is likely that in the near future a number of molecular markers will be identified which are linked to traits of commercial importance in silkworm. In the foreseeable future, the molecular marker assisted selection might play a key role in the improvement of silkworm breeds.

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Induction of premature mitosis in BHK cells blocked in S-phase

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Abstract

Induction of premature mitosis and its progression were studied in 2-aminopurine treated BHK_{21} cells blocked in S-phase by prior treatment with hydroxyurea. The cells showed premature induction of mitosis with nuclear envelope breakdown and chromosome condensation in general, and failed to progress through metaphase and anaphase. Immunofluorescence studies using anti-beta-tubulin antibody revealed normal spindle formation, but the chromosomes were not aligned to the spindle. Immunofluorescence and immunoelectron microscope studies using antikinetochore antibody showed that kinetochores got detached from the clumped and entangled chromosome mass possibly due to pull by the spindle fibres. Moreover during 2-AP treatment in S-blocked BHK cells no new cyclin is being synthesized and the initiation of mitosis is caused possibly due to activation of accumulated inactive mitosis promoting factor (MPF).

(**Keywords** : premature mitosis/metaphase/anaphase/animal & plant cells)

Introduction

Mitosis depends on the completion of S-Phase. However, Schlegel and Pardee[1] were first to report that this dependence can be overcome in BHK-cells by some purine derivatives which likely act as tyrosine kinase inhibitors[2]. Earlier several genetical mutants capable of overriding S-Phase dependency were reported[3,4]. Subsequently induction of premature mitosis has been reported in a number of animal cells[1, 5-9]. We demonstrated[10] that caffeine and 2-aminopurine (2-AP) could induce premature mitosis in S-blocked root metristematic cells of *Allium cepa*, indicating some common biochemical pathways leading to mitotic initiation in plant and animal cells. Schlegel and his co-workers[7] reported premature induction of mitosis in 2-AP treated BHK (baby hamster kidney) cells. Andreassen and Margolis[11] noted partial mitosis in these cells without functional spindle and thus the cells failed to enter metaphase or anaphase. Earlier Brinkley and co-workers[11] observed detachment of kinetochores from chromosomes in such prematurely induced mitotic cells. In onion

cells we[10] observed regular mitosis with functional spindle but with extensive fragmentation of chromatics at anaphase stage. This led us to undertake experiments using BHK_{21} cells to understand how the mitotic progression differed in animal and plant cells. Our results have been discussed in relation to our observation in onion cells to explain the difference in the mitotic progression in these two different cell types.

Materials and Methods

Baby hamster kidney (BHK_{21}) cell were used as material. Cells grown on tube slips were used for light microscopic and immunofluorescence studies. Cells were synchronized by treating them with 2.5 mM hydroxyurea (HU)[13] for 3 hrs. To induce premature mitosis BHK_{21}cells were treated with 2-aminopurine (Sigma) for different time periods at a concentration of 1 mM. For light microscopy cells were fixed in a solution of formaldehyde, acetic acid and alcohol mixed in the proportion of 15:1:85 and stained with haemalum. Cell spreads were prepared by treating them with 0.075 M KCl for 20 mins. Chromosomes were stained with Giemsa solution.

For light microscopic immunofluorescence studies cells were fixed in 4% freshly prepared paraformaldehyde and quenched in 50 mM NH_4Cl solution and permeabilized in 0.3% Triton-X-100 (containing 0.1% BSA and 0.1 gelatine dissolved in PBS) for 5 to 10 mins (for details see[14]). For localisation of spindle microtubules anti Beta-tubulin (1:100, monoclonal, Amersham) and Rhodamine linked goat anti-mouse IgG (1:50, Dianova) were used as primary and secondary antibodies. For detection of centromeres antibody obtained from a CREST patient was used in dilution 1:5000. Rhodamine linked anti-human antibody (Dianova) was used as secondary antibody. For detection of mitotic cyclin, anti-cyclin B_1 (1:5, monoclonal, Oncogene Science) and Rhodamine conjugated goat anti-mouse- IgG (1:50, Dianoval) were used.

For immunoelectronmicroscopy cells were fixed in 4% paraformaldehyde for 10 mins, quenched in 50mM NH_4Cl. Then the immunoreactions were performed. Anticentromeric antibody obtained from the serum of a CREST patient was used as the primary antibody (for 2 hrs.). 5 nm nanogold labelled antihuman IgG, raised in goat was used as secondary antibody (Nanoprobes INC, USA, for details of methods see[15]).

For immunoblot preparations cells were harvested both from the S-blocked and 2-AP treated cultures. Monoclonal anticyclin B_1 (Oncogene Science) was used as primary antibody and alkaline phosphatase conjugated goat anti-mouse IgG (Dianova) was used as secondary antibody. For details of procedure see[14].

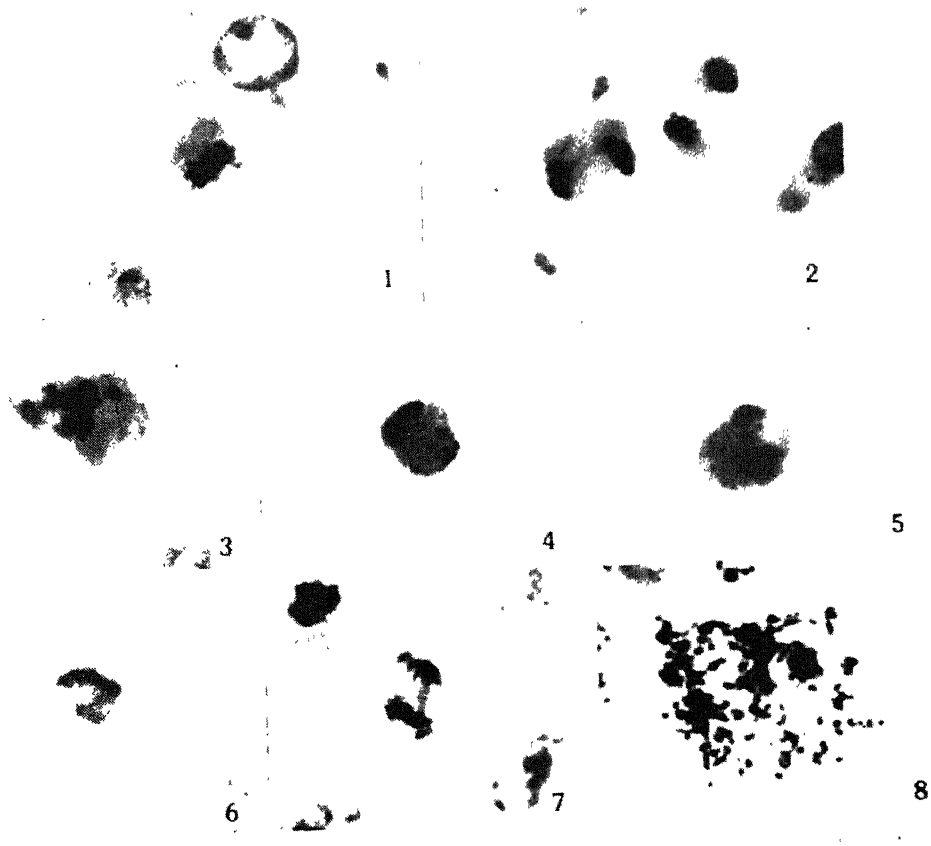

Fig. 1–2 : Control preparations from normal BHK_{21} cells.

1—a metaphase plate with chromosomes arranged at the equitorial plate.

2—cell in early and late telophase.

Fig. 3–8 : Cells blocked in S-phase induced to premature mitosis treated with 2-AP.

3—condensed chromosomes in a single clump.

4 and 5—showing more than one clump.

6—chromosomes in a single clump-note small bits of chromatin outside.

7—an anaphase appearing at the early stages of treatment.

8—a spread preparation from a prematurely induced mitotic cell-note thicker and thinner regions of the chromosomes.

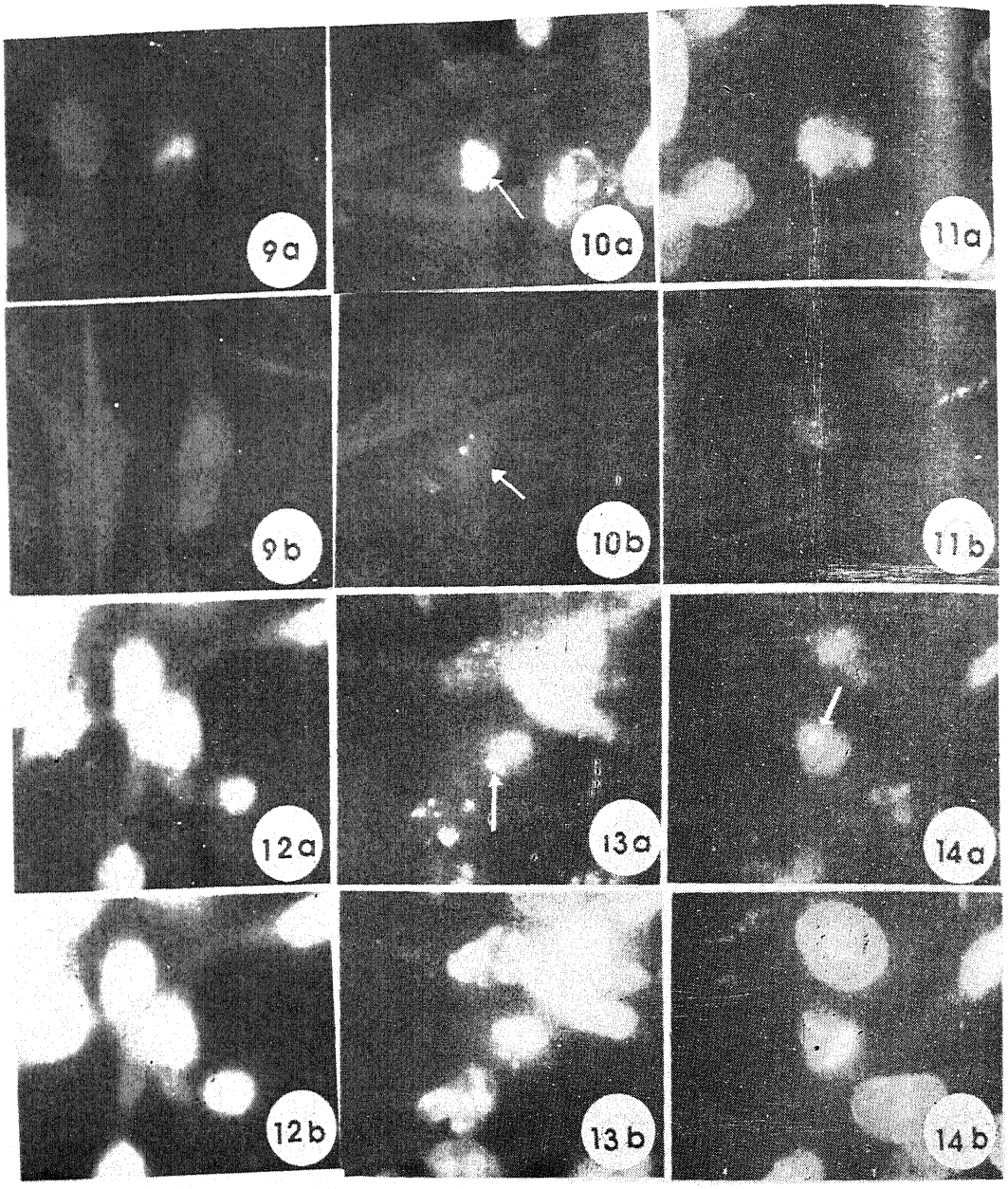

Fig. 9 : A normal mitotic cell immuno-stained for β-tubulin

(a) mitotic spindle (b) stained with Hoechst 33258.

Fig. 10 and 11 : Prematurely induced mitotic cells (a) showing mitotic spindle, (b) the same cell stained with Hoechst-note orientation of the spindle outside the main chromosome mass, only small bits of chromatin can be found within the spindle.

Fig. 12 : A normal mitotic cell

(a) immunostained for kinetochores

(b) the same cell, chromosomes stained with Hoechst.

Fig. 13 and 14 : Prematurely induced mitotic cells

(a) showing kinetochores

(b) the same cells stained with Hoechst-note kinetochores are located outside the main chromosome mass.

Results

The cells treated with hydroxyurea for 3 hrs. showed about 60% cells in S-phase (determined by flow cytometry-data not presented). These treated cells when allowed to recover in fresh medium free of drug showed normal mitosis after 5-6 hrs. of recovery. On the contrary, when the cells were continuously treated with HU for this period, no mitotic cell could be observed. However, the cells which were traded with HU for 3 hrs and subsequently treated with 2-aminopurine (2-AP) along with HU for different periods showed mitosis at different frequencies. The frequency increases with the period of treatment with 2-AP. Further experiments were done with cells treated for 8 hrs. which showed maximum frequency of mitotic cells (about 6%).

Light microscopic observation from haemalum stained control preparations revealed normal mitosis (4.2 %) with normal mitotic configurations (Figs. 1 and 2). Regular cytokinesis could also be observed. But in S-blocked cells treated with 2-AP, some of them rounded up and entered into mitosis. Condensed chromosomes could be seen in one (Fig. 3) or more clumps (Figs. 4 and 5), sometime bits of chromatin could be found in between. Small bits of chromatin could also be observed outside a prominent single chromosome clump (Fig. 6). Occasional metaphase and anaphase configuration could be found at the early period of treatment (3 to 4 hrs) (Fig. 7), but at later stages, such configuration could not be observed.

These mitotic cells in S-blocked population were induced to mitose prematurely became evident from spread preparations (Fig. 8). Thicker and thinner regions may represent the replicated ant unreplicated regions of the chromosomes.

The spindle morphology was studied by indirect immunofluorescence. Figs. 9(a, b) show normal spindle formation in a control cell. In treated cells the chromosome masses were found outside the spindle, but the spindles seemed to be in normal position. Only a small mass of chromatin could sometime be found within the spindle (Figs 10, 11).

The control preparations for immunofluorescence studies on kinetochores showed kinetochores in association with the chromosome mass as expected (Fig. 12). In prematurely induced mitotic cells the kinetochores were seen not in physical contact with the main chromosome mass (Figs. 13, 14) but in association with small bits of chromatin within the spindle region. The kinetochores seemed to be detached from the chromosomes. This can be better substantiated from immunoelectron microscopic pictures. Immunolabelled kinetochores can be seen outside main chromatin mass but associated with small chromatin bits aligned to the spindle poles (Fig 15). It can be noticed that some of the kinetochores have even moved to the poles (Fig. 16).

Immunoblots from the protein samples showed that the antibody had a higher affinity for the HeLa cyclin than the BHK_{21} cells. This is expected as the antibody was raised against human cyclin B_1. In BHK_{21} cells, the S-blocked cells and those subsequently treated with 2-AP did not show any difference in the cyclin B_1 level (Fig. 17) indicating no new synthesis of cyclin B_1 in the 2-AP treated cells.

Discussion

2-AP induces premature mitosis in BHK_{21} cells blocked in S-phase :

Baby Hamster kidney (BHK_{21}) cells treated with 2-AP in presence of hydorxyurea show premature mitosis in about 6% cells. The chromosomes from spread preparations show fuzzy, pulverized appearance comparable to that observed by Rao and Johnson [16] in their experiments fusing mitotic cells with S-phase cells. These prematurely induced mitotic cells show cytoplasmic 'rounding up' and nuclear envelope breakdown. Mitotic progression can be noted only in few cells which enter mitosis in early phase of treatment, but the cells entering mitosis later can not progress through metaphase and anaphase. Chromosomes formed clumps possibly due to stickiness of unreplicated chromosome segments and breaks caused by spindle pull.

Abnormal alignment of Chromosomes in relation to spindle :

2-AP induced premature mitotic cells exhibit chromosome condensation and other mitotic features, but the cells fail to progress through mitosis (also reported earlier[11,17]). However, spindle formation is normal in these cells, as can be seen by immunofluorescence study using beta-tubulin antibody. Clumped chromosome masses are found outside the spindle region. Further studies using antikinetochore antibody at the light microscope and electron microscope level reveal kinetochores with fragments of chromatin detached from the main chromosome mass. These kinetochores, however, are arranged within the spindle. Some of them even move towards the pole. Brinkley and co-workers[12] also observed this phenomenon.

No change in cyclin B level in cells blocked in S-phase and in cells subsequently treated with 2-AP :

Immunoblot results show that there is no significant variation in cyclin B level in HU blocked cells and cells treated simultaneously with 2-AP for another 8 hr. Earlier Steinmann and co-workers[18] noted that cyclin B synthesis was completed within S-phase in BHK cells. This is in agreement with present observations. In onion cells along with dephosphorylation of inactive MPF further synthesis of cyclin B is

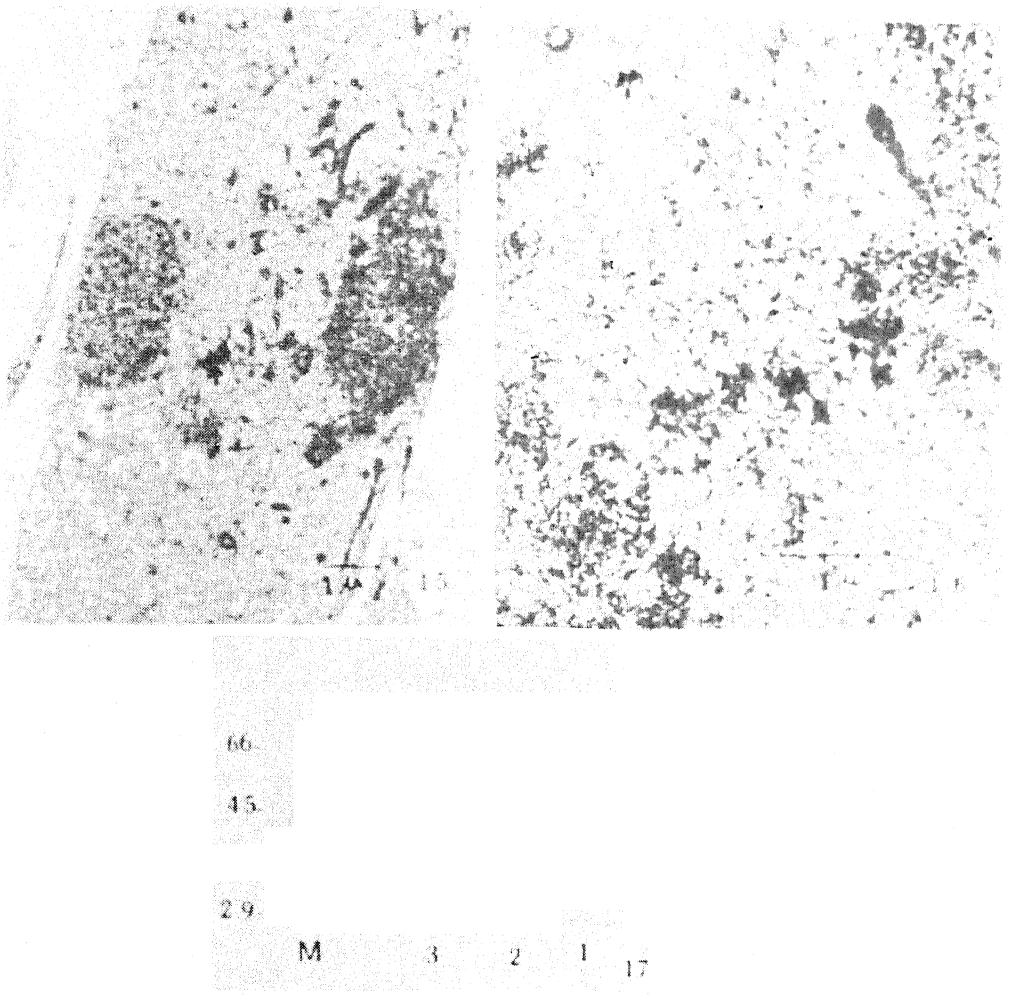

Fig. 15 : An electromicrograph of a prematurely induced mitotic cell in low magnification-note two main chromosome groups lying outside and the spindle with two centrioles lying in between. Bits of chromatin with detached kinetochores are attached to spindle fibres (arrows). Arrowhead points to a detached kinetochore almost reaching the pole.

Fig. 16 : Electron micrograph of a prematurely induced BHK_{21} mitotic cell at higher magnification showing gold labeling of the detached kinetochores (arrow).

Fig. 17 : Immunoblot analysis of cyclin B of whole cell extract of BHK_{21}.

Lone 1 – HeLa cells

Lone 2 – S-blocked cells treated with 2-AP for 8 hrs.

Lone 3 – Cells blocked in S-phase

M–Molecular weight markers.

necessary to initiate premature mitosis. But in BHK cells there is no significant amount of new cyclin B synthesis in 2-AP treated cells. Perhaps there is only activation (dephosphorylation) of accumulated MPF which leads to the induction of premature mitosis in these cells.

Kinetochore detachment is a consequence of premature induction of mitosis in S-blocked cells :

A few BHK_{21} cells show normal progression of mitosis in early period of 2-AP treatment, but afterwards these premature induced mitotic cells fail to show mitotic progression. These chromosomes form clumps and the kinetochores get detached possibly due to pull of the spindle fibres. Some of the detached kinetochores can even reach the poles. This is in contrast to what we observed in onion cells. Perhaps in *A. cepa* only late S-phase cells could enter premature mitosis. As such those cells could progress through mitosis, although high degree of chromosomal breaks could be observed in anaphase, due to failure of separation of sister chromatids in unreplicated regions of chromosomes[10].

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Zanthoxylum alatum extract as mosquito larvicide

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Abstract

Petroleum ether extract of *Zanthoxylum alatum* showed significant insecticidal activity against late third instar larvae of mosquito, *Culex pipiens quinquefasciatus* Say, LD_{50} of its active principle (fatty acids) for third instar mosquito larvae was found to be 20.45 ppm.

(**Key word :** toxicity/*Zanthoxylum alatum*/extraction/bioassay/fatty acids.)

Introduction

Chemical insecticides have been used indiscriminately during the past few decades to control the pests. This has produced serious repercussions such as pest resistance, mammalian toxicity, bio accumulation and environmental damage[1]. There are a number of plants, which have unique capacity to biosynthesize chemicals that are toxic to pests. Such Eco friendly phyto chemicals are termed as "bio pesticides" and the same are employed in the integrated pest management [2a, 2b]. We have been screening a number of plants as part of our endeavor to evaluate and identify bio active compounds. During bio-assay directed investigation on *Zanthoxylum alatum* (N.O. Rutaceae), we have identified an active fatty acid component from the petroleum ether extract which showed significant toxicity against larvae of mosquito, *Culex pipiens quinquefasciatus* Say. The present communication describes for the first time the isolation, identification, characterisation and evaluation of the insecticidal component of *Zanthoxylum alatum.*

Materials and Methods

Bioassay : A standard culture of mosquito was maintained in the laboratory at 27 ±°1C and 75 ± 5% RH. Extract was solubilised in a minimum amount of acetone. Water samples (50 ml) were treated with varying concentrations of *Zanthoxylum*

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alatum extract (50 to 400 ppm) and its fractions (varying from 10 to 75 ppm) were used for bioassay. About 20 third instar larvae were added in 50 ml of treated water for 24 hr. and insecticidal action was assayed by scoring percent mortality of the larvae. The experiments were repeated for 4 times with 20 larvae per replicate. Percent larval mortality was corrected using Abbott's formula[3]. The methods adopted for testing the extracts are based on procedures recommended by the World Health Organization which are basically modified Busvine and Nash method (1953) to score insecticidal activity[4].

Extraction and isolation of the insecticidal principle : The ground seeds (200 g.) of *Zanthoxylum alatum* were extracted with petroleum ether (60-80°) in a Soxhlet apparatus. The solvent was removed under vacuum to yield an oily residue (34 g.). The defatted seeds were further extracted with chloroform followed by the extraction with methanol. The above extracts were evaporated in vacuum to obtain respective crude extracts. A part of the residue of the petroleum ether extract (5 g.) was dissolved in ether and extracted with aqueous sodium hydroxide solution (2%). The aqueous phase was acidified with dilute hydrochloric acid and extracted with chloroform. The latter extract was washed with water, dried over anhydrous sodium sulphate and evaporated in vacuum to yield a mixture of fatty acids (1.8 g.). A part of this acid mixture was esterified with dry methanol and *p*-toluene sulfonic acid. The methyl esters were analyzed by GLC. The major components were identified as the esters of palmitic (16:0), 10.5%; palmitolic (16:1), 31.47%; *cis*-10-octadecenoic (18:1), 25.5%; *cis*-9.12-octadecadienoic, (18:2), 18.76% and *cis*-9,12,15-octadecatrienoic, (18:3), 12.65%, acids.

The toxicity studies were carried out with this fatty acid mixture obtained from the petroleum ether extract.

Results and Discussion

Table 1– Toxicity of extracts/fractions from *Zanthoxylum alatum* against third instar larvae of mosquito, *Culex pipiens quinquefasciatus* Say

Extracts/fractions	LD_{50} value (ppm)
Petroleum extract	315.50
NaOH extract	145.65
Fatty acid mixture	020.45

Zanthoxylum alatum is reported to have anti fungal[5], anti microbial[6], anthelmintic[7] and other medicidal properties[8]. In this paper for the first time its insecticidal activity against the mosquito larvae has been revealed. Fractionation protocol for the isolation of the insecticidal principle of this plant is presented in Fig.1. The LD_{50} value of each fraction has been presented in Table 1. It was observed that the portion containing fatty acid exhibited higher insecticidal activity against mosquito larvae as compared to other fractions. Upon probit analysis of this data, the LD_{50} was determined to be 20.45 ppm for this fraction. There are a number of reports such as oleic, caproic and octanoic acids which exhibit significant ovicidal as well as insecticidal activities[9-11].

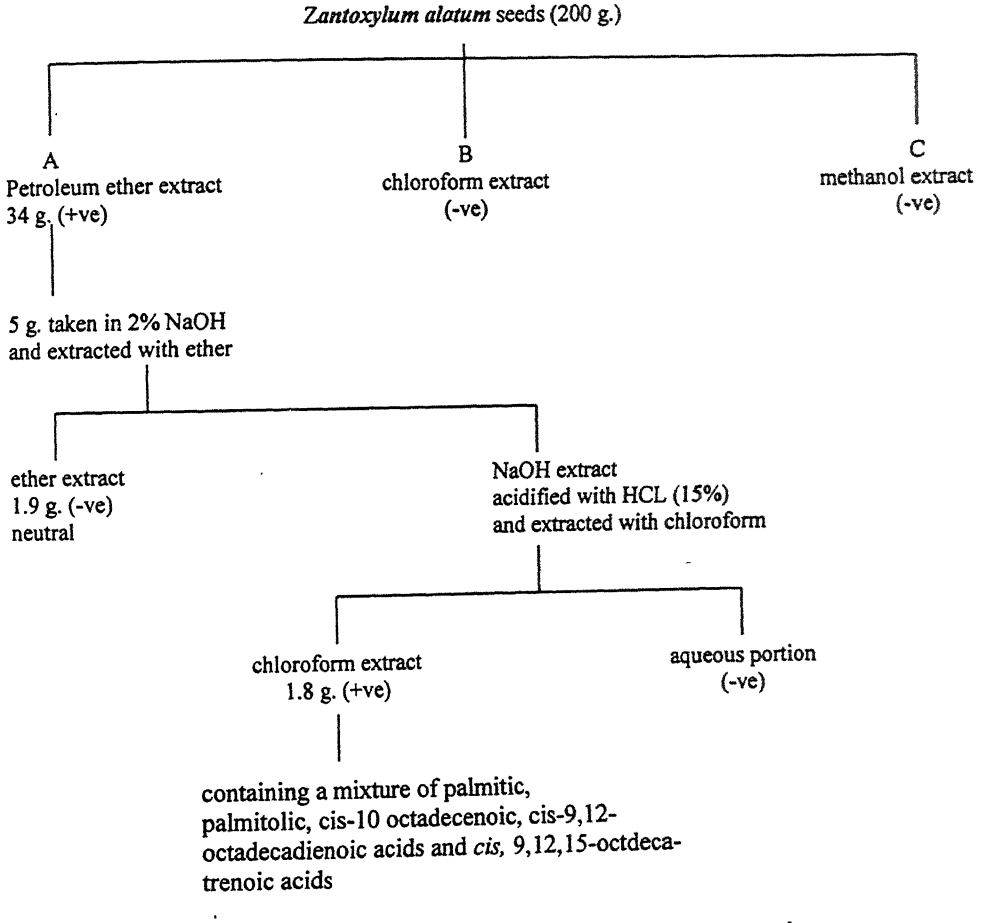

Fig. 1–Scheme for isolation of the active insecticidal principle of plant *Zanthoxylum alatum.*

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Bio-efficacy of *Clerodendrum siphonenthus* (Verbenaceae) against pulse beetle *Callosobruchus chinensis* (Coleoptera : Bruchidae)

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Abstract

The foliar part of *Clerodendrum siphonenthus* is dried at room temperature and extracted with petroleum ether, chloroform and methanol. Different concentrations are made from each crude extract and applied against *Callosobruchus chinensis* on filter paper. Observation is taken after 24 hours and its lethal concentration is calculated. Out of all the extracts, chloroform extract has shown highest mortality. The calculated LC_{50} values of chloroform extract is 2.81 mg/ml, methanol extract is 2.95 mg/ml and petroleum ether is 3.46 mg/ml. Several fractions (200 ml each) are collected through column chromatography from the chloroform extract and out of these fractions, fraction VII and XI are found to be highly effective. Thin layer chromatography is done with these fractions using toluene : ethanol : chloroform (50:50:50). The R_f value of fraction VII (Green) is 0.89 and fraction XI (pale yellow) is 0.71.

(**Key words :** *Clerodendrum siphonenthus/Callosobruchus chinensis*/insecticide/bio-efficacy).

Introduction

Due to increased public awareness about immediate and long term hazardous effect of synthetic pesticides, botanical insecticides are presently getting increased attention because they are environmentally safe, bio-degradable and leave no toxic residues in soil, food and fodder[1]. It has potentiality to preserve food grains like pulses which are heavily infested by stored grain pests during storage[2].

Clerodendrum siphonenthus R. BR (Verbenaceae), a tall erect shrub is locally known as Barangi in Hindi and Akalbih in Assamese[3]. the shrub has hollow stem and ashy grey, smooth bark. The flower is white in colour and fruit is 1.3 cm across, dark bluish green when ripe and seated on the enlarged red calyx. Its distribution is quite extensive. In India it is found from Madras (Chennai) in the South to Sikkim in the north and to Assam in the east. According to literature it is found from Tennesse in

USA to Sumatra in the far east[4,5]. The medicinal value of *C. siphonenthus* has been reported by many authors[6,7,8]. Some information is available on the isolation of a rice weevil feeding inhibitor from this plant. The two compounds uncinatone and pectoglinarigenin are reported from the fraction of petroleum ether leaf extract which shows inhibitory activity[9]. The two flavones, pectolinasigenin (I) and Cirsimartin (II) have been isolated from *C. siphonenthus* by column chromatography[10].

The use of *C. siphonenthus* has generated a lot of scientific interest because of its reported insecticidal activity. Besides there are reports of many plants showing insecticidal activity against insects[11,12,13].

Some information is available about the chemical components of certain parts of *C. siphonenthus*[5], for instance the bark of *C. siphonenthus*[5] contains hexitol (mannitol) along with sorbitol[5].

In the present experiment attempt has been made to assess the chemical which is responsible for showing insecticidal property against the stored product pest *Callosobruchus chinensis*.

Materials and Methods

Foliar extract of *Clerodendrum siphonenthus* :

C. siphonenthus leaves were collected from the field and dried at room temperature. The dried leaves were ground and extracted completely with petroleum ether (60-80 °C) for 4 days by cool extraction process. The same leaf materials were air dried and again extracted with chloroform for 4 days. Likewise the same material was dried and again extracted with methanol for 4 days. The extracts were then dried at room temperature in a vacuum desiccator.

Insect Rearing :

The pulse beetle *Callosobruchus chinensis* was collected locally and cultured in 1 kg container containing green gram at room temperature.

Bio-efficacy Testing of Extracts :

The petroleum ether (60-80 °C), chloroform and methanol extracts were diluted to different concentrations in ethanol (2.0, 2.25, 2.50, 2.75, 3.0, 3.50, 3.75, 4.00, 4.50 mg/ml) separately. Their 1 ml aliquots were sprayed on petriplate preloaded with Whatmann filter paper and then allowed to dry for 30 minutes at room temperature. In each plate 10 adult pulse beetles *Callosobruchus chinensis* were released after half

an hour and its activity and mortality were observed after 24 hours. Each test was done with three replications.

Fractionation of the crude extract and bioefficacy testing : The chloroform extract (2 gm) was run through column chromatography with the solvent petroleum ether and ethyl acetate in various ratios (10:0, 9:1, 8.2 and so on) and lastly with pure ethyl acetate. Thus several fractions (each of 200 ml) were collected and all the fractions were applied against *Callosobruchus chinensis*. Each fraction was diluted in ethanol and sprayed on filter paper and dried for half an hour at room temperature and the insects were slowly introduced in each petridish for assessing the active fractions.

Results and Discussion

The observation show that chloroform extract of *C. siphonenthus* leaf is more effective than methanol and petroleum ether extract. The insecticidal activity of *C. siphonenthus* and evaluation of LC_{50} is shown in Table 1, 2 and 3. The calculated lethal concentration. (LC_{50}) of petroleum ether extract is 3.46 mg/ml (Table 1), chloroform extract is 2.81 mg/ml (Table 2) and methanol extract is 2.95 mg/ml (Table 3). The chloroform extract was passed through column chromatography and several fractions are obtained. Of these, fraction VII was most effective (80% mortality), as also fraction XI (50% mortality) as compared to others (Table 4). The effective fractions were run in TLC using the solvent toluene : ethanol : chloroform (50:50:20). Two pure spots were observed. One single spot of green colour was observed in fraction VII having R_f value 0.89 and another single spot of pale yellow colour was observed in fraction number XI having R_f value 0.71.

From the present investigation it can be suggested that chloroform leaf extract is highly effective at low concentration (2.81 mg/ml) as compared to other extracts. The insecticidal application has been reported to show inhibitory effect on rice weevil also and the compounds isolated were uncinatone and pectolinasigenin from *C. siphonenthus*[9]. It is reported that the light petroleum ether (60-80 °C) extract on chromatography over silica gel affords a light yellow crystalline solid from the benzene ethyl acetate (9:1) elutes. The compound was identified as pectolinarigenin (I) (mp 200°C) which was earlierly isolated from *C. phlomids* and *C. inerme*[10]. In the present experiment a yellow spot was obtained from fraction XI of the chloroform extract of leaf of *C. siphonenthus* when run in TLC using the solvent tolune : ethanol : chloroform (50:50:50). This fraction however had caused 50% mortality of *C. chinensis* (Table 4). Fraction VII which gave a single green spot on TLC was most effective causing 80% mortality in these beetles.

Table 1– Effect of Petroleum ether extract of *Clerodendrum siphonenthus* Leaf against *Callosobruchus chinensis* and evaluation of LC_{50}.

Conc. (mg/ml)	Total No. of Insects	Number of dead Insect	% of Mortality	Corrected Mortality	Logdose	Probit
2.00	30	2	6.6	7	0.301	3.52
2.25	30	5	16	16	0.352	4.01
2.50	30	9	30	30	0.397	4.48
2.75	30	12	40	40	0.439	4.75
3.00	30	14	46	46	0.477	4.90
3.50	30	16	53	53	0.544	5.08
3.75	30	18	60	60	0.570	5.25
4.00	30	22	73	73	0.602	5.61
4.50	30	26	86	86	0.650	6.08

The calculated LC_{50} Value is 3.46 mg/ml.

Table 2– Effect of Chloroform extract of *Clerodendrum siphonenthus* Leaf against *Callosobruchus chinensis* and evaluation of LC_{50}.

Conc. (mg/ml)	Total No. of Insects	Number of dead Insect	% of Mortality	Corrected Mortality	Logdose	Probit
2.00	30	9	30	30	0.301	4.48
2.25	30	10	33.3	33	0.352	4.56
2.50	30	12	40	40	0.397	4.75
2.75	30	14	46.6	47	0.439	4.92
3.00	30	19	63.3	63	0.477	5.33
3.50	30	20	66.6	67	0.544	5.44
3.75	30	23	76.6	77	0.570	5.74
4.00	30	25	83.3	83	0.602	5.95
4.50	30	29	96.6	97	0.650	6.88

The calculated LC_{50} Value is 2.81 mg/ml.

Table 3.– Effect of Mehanol extract of *Clerodendrum siphonenthus* Leaf against *Callosobruchus chinensis* and evaluation of LC_{50}.

Conc. (mg/ml)	Total No. of Insects	Number of dead Insect	% of Mortality	Corrected Mortality	Logdose	Probit
2.00	30	2	6.6	7	0.301	3.52
2.25	30	5	20	20	0.352	4.16
2.50	30	8	26.6	27	0.397	4.39
2.75	30	10	33.3	33	0.439	4.56
3.00	30	15	50	50	0.477	5
3.50	30	17	56.6	57	0.544	5.18
3.75	30	19	63.3	63	0.570	5.33
4.00	30	20	66.6	67	0.602	5.44
4.50	30	25	83.3	83	0.650	6.95

The calculated LC_{50} Value is 2.95 mg/ml.

Table 4– Percentage of mortality of *C. chinensis* in some constituent fractions of chloroform extract of *Clerodendrum siphonenthus* Leaf.

Dose	Fraction	% of Moratality
0.5 mg	I	20
0.5 mg	V	30
0.5 mg	VI	30
0.5 mg	VII	80
0.5 mg	X	20
0.5 mg	XI	50

Thus it may be concluded that *C. siphonenthus*, is a locally available plant having great potentiality as a botanical insecticide.

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Propagation of *Coelogyne breviscapa* Lindle and *Coelogyne nervosa* A. Rich through asymbiotic seed germination

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Abstract

Seeds of *Coelogyne breviscapa* Lindl and *C. Nervosa* A. Rich were asymbiotically cultured on four different basal media. MS medium was effective in inducing seed germination in both the species. IAA enhanced seedling growth in *C. breviscapa* and NAA in *C nervosa*. Among the cytokinins BAP significantly enhanced seedling growth compared to the control. Incorporation of coconut water and banana pulp in the medium did not affect the seedling growth significantly. Yeast extract (3mg/l) was found to be effective for seedling growth in *C nervosa* and peptone (3mg/l) in *C breviscapa*.

(**Key words :** *Coelogyne breviscapa/C. nervosa*/asymbiotic seed germination).

Introduction

Orchids constitute an order of royalty in the world of ornamental plants and they are of immense horticultural importance and also play a very useful role to balance the forest ecosystems[1]. They occupy top position among all flowering plants valued for cut flower production and as potted plants, which fetch a very high price in the international market. The most common method of propagation of orchids is by division of pseudobulbs, cuttings and air layering. The *in vitro* techniques in orchid propagation have revolutionized orchid industry and resulted in the large number of uniform plants obtained within a short period for successful establishment of orchid industry.

In recent times, the wild orchids are also commercially exploited for horticultural trade. Although these species are still common, the development of an artificial means of seed propagation is needed to reduce collection pressures on wild population. Both tissue culture and seed propagation are options which could be used to rapidly increase plant numbers. Seed propagation may provide the best alternative, since it would conserve genetic variability. Hence the present study was attempted for mass

propagation and establishment of *Coelogyne nervosa.* endemic to western ghats and *Coelogyne breviscapa.*

Materials and Methods

The undehisced capsules (approximately 180-250 days old) of *Coelogyne nervosa.* A. Rich. and *Coelognye breviscapa* Lindl. were collected from Naduvattam evergreen tropical forest at an altitude of 1800 meter above MSL and Mulakkadu evergreen tropical forest at an altitude of 1500 meter above MSL, Nilgiri district, respectively.

Four different basal media namely Knudson C[2], Murashige and Skoog[3], Vacin and Went[4] and B5[5] were initially used for seed germination. The pH of all the media was adjusted to 5.8 prior to adding agar, 0.8% agar (Hi-media) was used as gelling agent. The prepared medium was dispensed in 25 × 30 mm borosil test tubes. The mouth of the test tubes were plugged with aluminium foil and autoclaved for 25 minute at 121 °C and 15 lb/in pressure. All the cultures were maintained under 12:12 h photoperiod (3000 lux light) at 25±2 °C and 70% relative humidity. After 10 days of inoculation, 20 samples were selected at random and observed under microscope and the germination percentage was calculated.

After 90 days the best medium was selected for further studies. The above medium was enriched with plant growth regulators, like NAA, IAA, BAP and KIN in different concentrations ranging between 0.2-1.0 mg/l and growth adjuvants like peptone, yeast extract (1-3 mg/l) and coconut water (5, 10 and 15%) individually to study their effects on the growth of the seedling. Three replicates were maintained for each treatment.

The well developed seedlings were removed from culture vessels and thoroughly washed with tap water to remove the adhering medium completely without causing damage to the roots. Then the plantlets were treated with the fungicide solution [Bevistin (carbendazim 50% WP)] at 5% concentration and transferred to plastic cups with the following media for hardening.

Vermiculate + brick pieces + charcoal + sphagnum moss in the ratio of 1:1:1:1.

and red soil + vermiculate + charcoal + sphagnum moss in the ratio 1:1:1:1.

The potting media were autoclaved and about 10 seedlings were placed in each cup. The hardening was maintained under low level polythene tunnels for well establishment.

Results

The seeds were aseptically removed and sown on four different basal media. Greening of the seeds were observed after four weeks in Knudson C medium followed by MS medium. The seed germination was delayed in Vacin and Went (fifth week) and B5 (sixth week) media. The germination percentage (60%) significantly decreased in B5 medium (Table-1a). During the germination the undifferentiated embryo emerged fro testa and formed green protocorms (Fig 1). The protocorms developed first leaf and root during the fifth week in Knudson C medium followed by MS medium (sixth week). Though the protocorm differentiated first in Knudson C medium, the length of the fist leaf, root and diameter of the protocorm in both the species were found to be more in MS medium. Hence, MS medium was selected further to study the effect of growth regulators and growth adjuvants on the development of seedlings.

Table 1a.– Effect of different basal medium on seed germination of *C. breviscapa* and *C. nervosa*.

	Coelogyne breviscapa				*Coelogyne nervosa*		
S. No.	Medium (Basal)	Time taken for germination	Time taken for protocorm formation	% of seed germination	Time taken for germination	Time taken for protocorm formation	% of seed germination
1.	MS	4 weeks	5 weeks	100	4 weeks	5 weeks	100
2.	KC	4 weeks	6 weeks	100	4 weeks	7 weeks	95
3.	V W	5 weeks	8 weeks	70	5 weeks	9 weeks	70
4.	B5	6 weeks	9 weeks	60	6 weeks	9 weeks	60

MS = Murashige and Skoog, KC = Knudson C

VW = Vacin and Went

Ninety day-old healthy protocorms obtained from MS medium were subcultured in the same medium containing different growth regulators individually at different concentrations. Among the auxins, IAA at 1mg/l increased the shoot and root length in *C. breviscapa* (Fig 2) whereas NAA at 1mg/l enhanced the shoot and root length in *C. nervosa.* The seedlings developed on MS medium supplemented with BAP (1mg/l) (Fig 3) were dark green in colour, and there was increase in the root and shoot length of both *C. breviscapa* and *C. nervosa.* Kinetin at the same concentration increase only the shoot length in both the species (Table 1b).

Table 1b–Effect of various growth regulators on seedling growth of *Coelogyne breviscapa* and *C. nervosa*.

Sl. No.	Medium growth regulator	Concentration (mg/l)	*Coelogyne breviscapa*		*Coelogyne nervosa*	
			Shoot length (cm) Mean±SE	Root length (cm) Mean±SE	Shoot length (cm) Mean±SE	Root length (cm) Mean±SE
1.	MS+NAA	0.2	1.2±0.035	0.4±0.022	1.2±0.07	0.25±0.07
2.		0.5	1.3±0.035	0.45±0.022	1.3±0.054	0.3±0.015
3.		1.0	1.4±0.042	0.5±0.023	1.4±0.027	0.35±0.035
4.	MS+IAA	0.2	1.5±0.027	0.45±0.041	1.2±0.035	0.3±0.015
5.		0.5	1.6±0.035	0.50±0.022	1.3±0.016	0.35±0.035
6.		1.0	1.7±0.035	0.55±0.017	1.3±0.054	0.3±0.035
7.	MS+BAP	0.2	1.3±0.035	0.40±0.022	1.2±0.070	0.25±0.015
8.		0.5	1.3±0.035	0.35±0.032	1.3±1.054	0.2±0.063
9.		1.0	1.5±0.022	0.45±0.022	1.4±0.044	0.3±0.035
10.	MS+KN	0.2	1.3±0.035	0.35±0.032	1.1±0.070	0.2±0.063
11.		0.5	1.4±0.035	0.40±0.022	1.2±0.035	0.2±0.035
12.		1.0	1.8±0.035	0.40±0.022	1.3±0.016	0.25±0.015

Values are mean (n = 10) ± SE

The well developed protocorms obtained from MS medium were subcultured on the same medium containing various organic supplements like peptone, yeast extract and coconut water. Among the different growth adjuvants, peptone (3mg/l) (Fig 4) enhanced the root and shoot length in *C. breviscapa* whereas in *C. nervosa* yeast extract (3mg/l) was found to be suitable for subsequent seedling growth. Incorporation of coconut water, irrespective of concentration, did not affect the seedling growth significantly (Table 2). About 80% of the *in vitro* raised seedlings were established in the potting medium containing vermiculate + brick pieces + charcoal + sphagnum moss in the ratio 1:1:1:1.

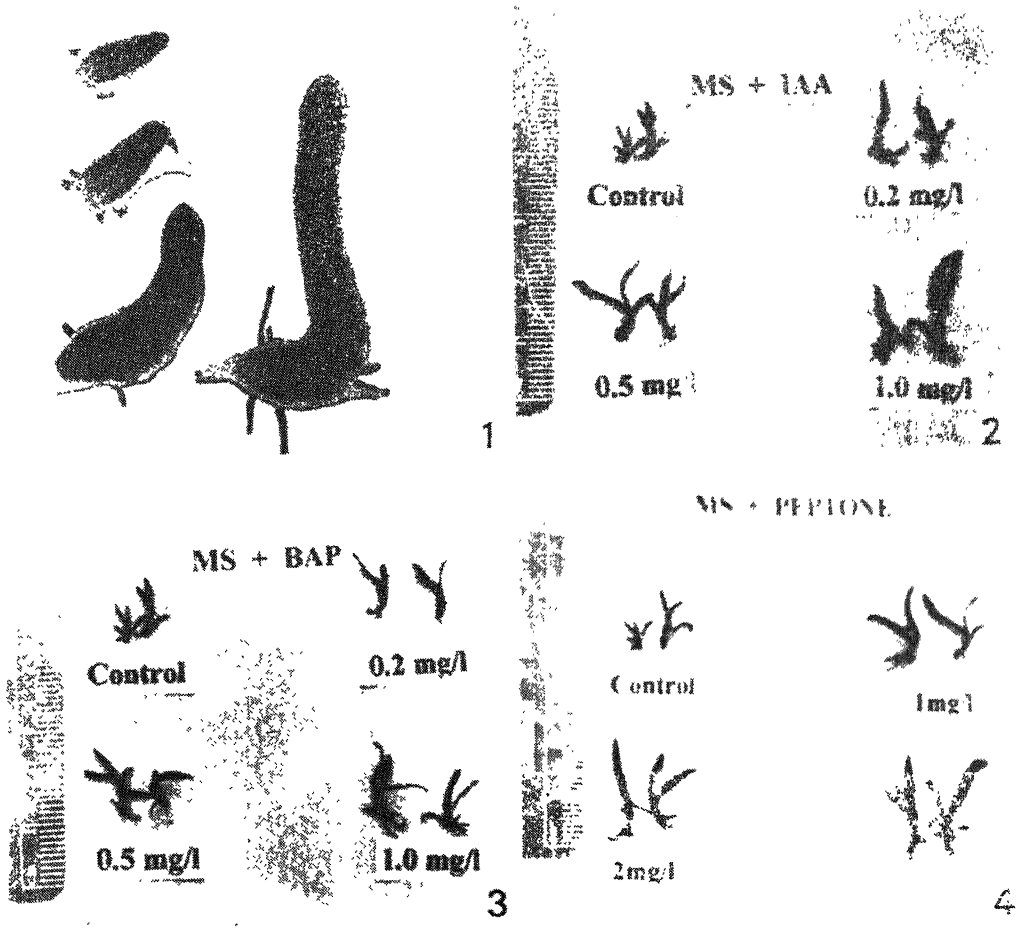

Fig. 1-4– *Coelogyne breviscapa*

1. Different stages in the development of protocorm.
2. Effect of IAA on seedling growth.
3. Same, BAP at different concentration.
4. Effect of Peptone on seedling growth.

Table 2.– Effect of growth adjuvants on seedling growth of *Coelogyne breviscapa* and *C. nervosa*

Sl. No.	Medium + growth adjuvants	Concentration	*Coelogyne breviscapa*		*Coelogyne nervosa*	
			Shoot length (cm) Mean ± SE	Root length (cm) Mean ± SE	Shoot length (cm) Mean ± SE	Root length (cm) Mean ± SE
1.	MS+peptone	1 mg/l	1.6±0.22	0.4±0.23	1.2±0.70	0.2±0.35
2.		2 mg/l	1.8±0.35	0.6±0.18	1.3±0.54	0.2±0.63
3.		3 mg/l	2.0±0.35	0.6±0.22	1.3±0.16	0.25±0.70
4.	MS+YE	1 mg/l	1.6±0.27	0.3±0.15	1.2±0.35	0.2±0.24
5.		2 mg/l	1.8±0.37	0.5±0.22	1.3±0.44	0.25±0.27
6.		3 mg/l	1.9±0.35	0.45±0.22	1.4±0.27	0.25±0.40
7.	MS+CW	5%	1.6±0.37	0.4±0.27	1.8±0.47	0.25±0.37
8.		10%	1.5±0.27	0.3±0.27	1.0±0.27	0.15±0.15
9.		15%	1.5±0.22	0.4±0.22	1.0±0.22	0.25±0.15

Values are mean (n = 10) ± SE

Discussion

Tissue culture method for regeneration of orchid were introduced by several workers using various explants and culture media. Among the various explants, the shoot tip and axillary bud were commonly used. The seed germination in orchids in nature is an unique phenomenon and require fungal association. Germination is much more successful *in vitro*. In the present study among four media tried, the seeds of both *C. breviscapa* and *C. nervosa* though germinated early in Knudson C medium, MS medium was found to be more suitable for the development of protocorms (Table 1a).

The nutritional requirement varies with the genus, species and locality[6]. Several author suggested different nutrient media suitable at different stages of growth for various species. In the present study seeds of both *C. breviscapa* and *C. nervosa* germinated and developed seedlings on all the media used. MS medium proved to be better for the development of protocorms.

Addition of growth regulators and organic supplements is needed in some species for the enhancement of seed germination and seedling growth. In majority of the cases auxins enhanced germination and seedling growth[7]. In the present investigation, NAA (1mg/l) was effective in promoting seedling growth in *C. nervosa* and IAA *C. breviscapa*. The effectiveness of these auxins for growth of orchid seedlings has been reported by many investigators[8,9] BAP and Kinetin had more or less the same effect on the development of seedling in both the species. Kinetin (1-10mg/l) retarded seed germination and seedling growth in *Coeloglossum viride* and *Plantanthera bifolia*[10].

Among the organic supplements employed, peptone at 3 mg/l in the medium enhanced the shoot length in *C. breviscapa* and yeast extract at 3 mg/l in *C. nervosa.* The shoot development was significantly affected by coconut water as reported in *Dendrobium chrysanthum* and *Paphiopedilum spicerianum*[11]. The *in vitro* seedlings were successfully established in potting medium. Further studies will be carried out to establish the *in vitro* raised seedlings in their original habitat.

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Mycofloral spectrum during storage and its effect on seed viability of soybean [*Glycine max* (L.) Merrill] seeds under ambient conditions

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Abstract

Soybean seed of JS-80-21 and Pusa-16 varieties were sampled and treated with mancozeb, thiram, numbecidine and bleaching powder and stored in two types of containers under ambient storage conditions. The percent occurrence of themophilic/thermotolerant flora was 29.8% as against 70.2% of mesophilic flora, irrespective of treatments. Amongst the two storage containers, polylined bag harboured low incidence of mycoflora (37.6%) as compared to cloth bag packaging (62.4%). Amongst seed treatments, mancozeb (78.6%) and thiram (65.1%) seed dressings controlled seed mycoflora more effectively than treatments with nimbecidine (10.1%) and bleaching powder (13.0%). With advancing storage period, the field fungi (*Cladosporium cladosporioides, Alternaria alternata, Acremonium* sp., *Fusarium moniliforme, F. semitectum. Curvularia lunata* and mycelia sterilia) were replaced with storage fungi (species of *Aspergillus, Pencillium, Rhizopus, Mucor, Chaetomium, Epicoccum* and *Trichothecium*). The reasons for appearance and non-appearance of seed mycoflora during storage can be attributed to the variations in temperature and RH of seed godown. A highly significant negative correlation (r= -0.8718) between seed viability and seed mycoflora suggest seed mycoflora to be an important factor responsible for seed deterioration during storage under ambient conditions.

(**Key words** : soybean/seed mycoflora/storage/seed viability/seed treatment).

Introduction

The role of seed mycoflora in seed spoilage is being increasingly recognized during recent years[1,2]. The loss due to fungi can be especially high under tropical and sub-tropical conditions where high humidity and temperature coupled with poor storage conditions exist and the mechanical injuries caused to the seed during harvesting facilitate easy entry of fungal pathogens during storage. The field and storage fungi virtually classified on the basis of the stage at which invasion and growth of fungi occur in/on the seed, play certain role in the deterioration of stored

products[3]. Temperature variations in the seed godown also play an important role in the development of the storage fungi. Broadly speaking, amongst the seed borne fungi, the primary invaders during storage may be either mesophilic or thermophilic. Thermophilic fungi may be present as superficial contaminants of grain before harvest but can invade grain kernels when spontaneous heating occur in storage, and therefore can be regarded as storage fungi. Proper storage and application of safe chemicals as post- harvest treatment can considerably reduce the losses in storage. The available literature indicates that the work done on storage losses in agricultural products is mainly confined towards understanding the ecology of seed mycoflora of any one group of microorganisms. The present work was initiated to study the mycoflora on soybean seed at different temperatures, their persistence in different treatments and their effect on seed viability during storage under ambient conditions.

Materials and Methods

The processed seed lots of two varieties of soybean- JS-80-21 and Pusa-16, harvested during December 1995, were sampled and treated with mancozeb 75% WP, thiram 75% DS and bleaching powder each @ 2.5/kg seed and with nimbecidine (azadirachtin 0.03 % EC)@ 2.5 ml/kg seed as dry dressing. One sample was left untreated, which served as control. One half of these samples were stored in cloth bag and the other half in polythene bag of 400 gauge inside a cloth bag, henceforth referred to as polylined bag. These subsamples were kept inside a seed godown at IARI, Regional Station, Karnal under ambient storage condition.

Storage conditions of seed godown :

The minimum and maximum temperatures and relative humidity of the seed godown was recorded daily using Heal's thermometer and Huger's Hygrometer respectively, from February 1996 to Feb., 1997. The minimum temperature of the seed godown varied from 13.4 °C to 37.5 °C during the same months. The relative humidity of the seed godown varied from 51.9 per cent in April 1996 to 75.4 per cent in January 1997 (Fig. 1).

Seed mycoflora :

The fungal flora associated with seeds in different treatments was studied following blotter test method. The seeded plates were incubated for 5 days at 45 °C to isolate themophilic fungi, and at 28 °C for 7 days to isolate mesophilic fungi. Percent occurrence of fungi was calculated as:

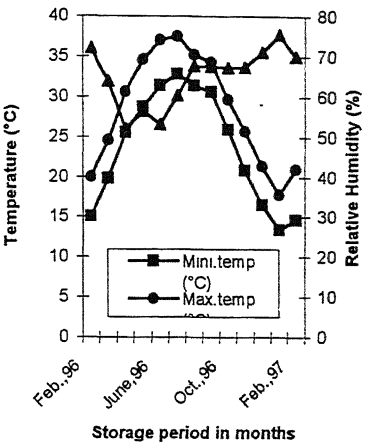

Fig. 1–Temperature and relative humidity of seed godown during storage.

$$\frac{\textit{Total number of seeds in which a particular fungus occurred}\ X\ 100}{\textit{Total number of seeds incubated}}$$

Average percent seed mycoflora control was calculated as :

$$\frac{\textit{Average incidence of seed mycoflora in control treatment} - \textit{Average incidence of seed mycoflora in fungicidal treatment}}{\textit{Average incidence of seed mycoflora in control treatment}}$$

The mycoflora found associated with soybean seeds in different treatments has been grouped into four categories—

Category I Seeds of JS-80-21 variety, stored in cloth bag packaging,

Category II Seeds of JS-80-21 variety, stored in polylined bag packaging,

Category III Seeds Pusa-16 variety, stored in cloth bag packaging,

Category IV Seeds Pusa-16 variety, stored in polylined bag packaging.

Results and Discussion

Ten species were recorded on the seeds in soybean 'cv' JS-80-21, under thermophilic/thermotolerant group and 19 species were recorded under mesophilic group (Table 1). In 'cv' P-16. 12 and 17 spp. were recorded under thermophilic/ thermotolerant and mesophilic groups respectively, irrespective of treatments. In all, five species of *Aspergillus* were found associated with seeds of P-16 variety, whereas *A. candidus* was found to be absent in JS-80-21 variety. *Trichoderma* sp. and mycelia sterilia were missing on the seeds of P-16 variety. *Trichothecium roseum* was recorded on the seeds of both the varieties stored in cloth bag packing only. The percent occurrence of *Cladosporium cladosporioides* as mesophile was maximum and it decreased with increasing storage duration whereas an actinomycete and *Aspergillus fumigatus* was recorded as thermophile at the time of storage. *A. flavus* and *Fusarium moniliforme* were also recorded as thermophile whereas *F. semitectum* and *T. roseum* occurred as mesophiles. The percent occurrence of thermophiles and mesophiles on the seeds of JS-80-21 variety was 47.3% as against 52.6% found associated with seeds of P-16 variety (Fig. 2). The percent occurrence of thermophilic/thermotolerant flora was 29.7% as against 70.2% of mesophilic flora, irrespective of treatments. The incidence of fungal species associated with seeds was low in polylined bag packaging (37.6%) as compared to 62.3% in cloth bag packaging. Also, 33.5% and 28.9% mycoflora was recorded in category I and III as against 13.8% and 23.8% in categories II and IV, respectively (Fig. 3). Further, 7.8% and 9.9% of thermophilic/thermotolerant species were recorded in categories I and III as against 4.9% and 7.3% in categories II and IV respectively. 25.7% and 19.0% of mesophilic flora was recorded in categories I and III as against 8.9 and 16.5% in categories II and IV, respectively. Though the incidence of thermophilous flora associated with soybean seeds was observed to be lower than mesophilic mycoflora but their importance during storage resulting in seed deterioration needs more exploration.

The rapid increase in the fungal population in cloth bag packaging during the initial phase of storage can evidently be attributed to the significant rise in RH during the rainy season coupled with increasingly favourable temperature (Fig. 1). The moisture content of seed varied from 5.5 to 11.1 per cent in different treatments. Arafa and co-workers[4] also observed that fungal invasion was greatest after four months of storage coinciding with highest seed moisture content, and on prolonged storage both the temperature and RH registered a comparative decline and hence the total population did not show any significant rise. Low fungal populations recorded on seed stored in polylined bag as against those stored in cloth bag, may be due to the closed atmosphere of the polylined bag, which does not allow the environmental fluctuations

to affect the seed and seed mycoflora. It may then gradually become rich in the CO_2 contents and may inhibit the fungal growth and population. Thus, RH and temperature of the seed godown might be responsible for seed deterioration during storage under ambient condition. Zagrebenyer and Bern[2] were also of the view that storage fungi were the major factor causing soybean seed deterioration.

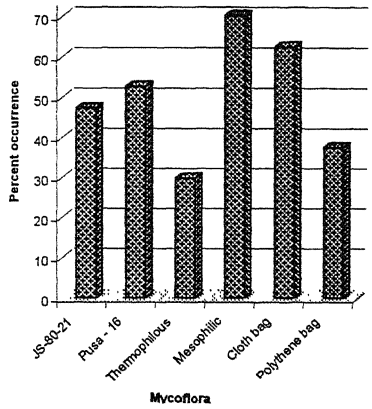

Fig. 2–Percent occurrence of mycoflora amongst varieties (JS-80-21 & P-16), groups (thermophilous & mesophilic) and storage containers (cloth & polylined bag).

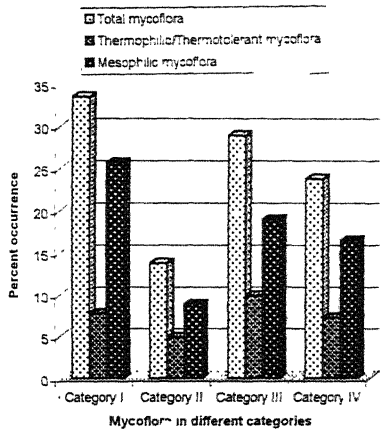

Fig. 3–Occurrence of total mycoflora, thermo-philic/ thermotolerant and mesophilic mycoflora in different treatments.

The effect of seed dressings was similar in both the varieties of soybean stored in both the containers, though percent occurrence of storage fungi especially, *A. flavus, A. niger, A. fumigatus, A. ochraceus* and *Rhizopus stolonifer,* were higher in categories I and III as against categories II and IV (Table 1). Kushwaha and Raut[5] had reported that the seeds treated with thiram and stored in polylined bag suppressed most of the fungi. Our studies also showed that seed treatment with maniozed and thiram reduced the incidence of fungi on seeds as against other seed treatments. Only 6.4 and 10 per cent of the total seed mycoflora was present associated with mancozeb and thiram treated seeds respectively, as against 26.9% in nimbecidine and 26.1% in bleaching powder treatments, which were almost at par with control (30.0%). On calculating the average percent seed mycoflora control, mancozeb (78.6%) was most effective during storage followed by thiram (65.1%), bleaching powder (13.1%) and nimbecidine (10.1%), indicating the ineffectivity of nimbecidine and bleaching powder in controlling seed mycoflora during storage as against the fungicides (Fig. 4). Asalmol and Zade[6] also observed that pre-storage seed treatment helps to improve the shelf life of seeds and checks seed mycoflora during storage.

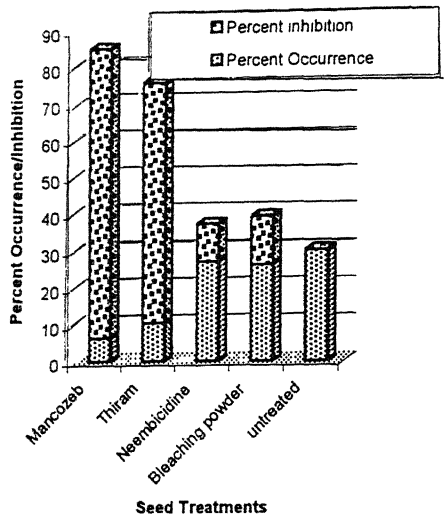

Fig. 4–Influence of seed dressings on percent occurrence and inhibition of seed mycoflora.

Persistence of mycoflora during storage :

The persistence of fungi/actinomycete on the stored seeds differed in different categories. The actinomycete was consistently present during storage on the seeds as thermophile. Amongst the fungi, *Aspergillus fumigatus* was most consistently found associated with the seeds of all the categories as thermophile. Species of *Penicillium, Rhizopus* and *Aspergillus* except *A. flavus* appeared later during the storage period and were absent initially at the time of storage. *A.flavus* was present during storage period. *Mucor* was initially absent in categories III and IV and irregular in appearance in categories I and II. Most of the mesophilic fungi like *Fusarium semitectum, Alternaria alternata, Curvularia lunata, Trichothecium roseum, Chaetomium spinosum, Epicoccum nigrum, Acremonium* sp. (=*Cephalosporium* sp.) and *Trichoderma* sp. were present irregularly on the seeds in different categories. The storage fungi like species of *Chaetomium, Epicoccum* and *Trichothecium* also appeared at a later stage during storage. The incidence of *Cladosporium cladosporioides, Alternaria alternata* and *Acremonium* sp. declined with increase in the storage duration, and thus they behaved as field fungi. Nakka[7] also observed a decline in the incidence of *Cladosporium* and *Alternaria* and increase in the incidence of *Aspergillus* and *Penicillium* with advancing storage period. The variations in the storage conditions i.e., temperatures and relative humidity of the uncontrolled seed godown, may be the reasons attributed for appearance and non-appearance of fungi during storage.

Table 1– Mycoflora found associatedwith soybean seeds in different categories at different storage periods.

Mycoflora	Feb.'96		June 1996				October 1996				February 1997			
	Cat I-II	Cat III- IV	Cat I	Cat II	Cat III	Cat IV	Cat I	Cat II	Cat III	Cat IV	Cat I	Cat II	Cat III	Cat IV
THERMOPHILIC/THERMOTOLERANT MYCOFLORA														
Fusarium moniliforme	-	1	6	4	4	27	1	-	5	2	10	22	45	21
Aspergillus niger	-	-	-	-	-	-	-	-	1	-	5	2	3	2
Aspergillus fumigatus	4	8	18	15	14	18	3	3	6	10	19	11	21	11
Aspergillus ochraceus	-	-	-	-	-	-	1	1	-	3	5	2	-	23
Aspergillus flavus	-	4	-	-	-	-	6	1	19	12	25	11	87	33
Aspergillus candidus	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rhizopus stolonifer	-	-	-	-	-	-	-	-	4	1	76	18	89	11
Penicillium sp.	-	-	-	3	12	7	5	2	-	-	-	11	-	21
Mucor pusillus	-	-	12	43	6	4	17	2	9	7	20	-	21	34
Mycelia sterilia	-	-	4	2	14	8	-	-	2	-	18	-	12	-
*Actinomycetes	16	-	145	66	95	61	98	45	91	55	54	88	34	65
Cladosporium cladosporioides	-	5	-	-	-	14	-	-	6	9	-	-	10	34
MESOPHILIC MYCOFLORA														
C cladosporioides	95	99	14	8	-	-	-	7	-	5	7	5	20	-
Alternaria alternata	2	-	5	7	3	-	-	-	-	-	13	12	6	27
Fusarium moniliforme	2	-	12	3	40	19	71	21	32	5	10	15	25	1
Fusarium semitectum	-	2	24	-	9	2	-	6	9	10	9	2	3	1
Aspergillus flavus	-	-	161	116	74	82	287	124	287	297	126	27	96	83
Aspergillus niger	-	-	66	7	50	49	50	25	4	12	42	18	15	34
Aspergillus fumigatus	-	-	-	-	-	-	2	-	-	-	-	-	7	10
Aspergillus ochraceus	-	-	-	-	-	-	43	8	30	20	12	-	10	12
Curvularia lunata	-	-	1	-	2	-	6	2	-	5	2	5	-	-
Acremonium sp.	-	-	-	-	-	-	-	-	-	-	-	-	1	1
Rhizopus stolonifer	-	-	9	7	5	4	390	89	236	95	241	43	181	136
Mycelia sterilia	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Penicillium sp.	-	-	47	11	68	64	35	18	8	32	-	21	29	17
*Actinomycetes	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Trichothecium roseum	-	6	-	-	2	-	2	-	-	95	-	-	-	136
Chaetomium spinosum	-	-	1	-	-	-	4	-	4	-	-	-	7	-
Epicoccum nigrum	-	-	-	-	-	-	-	-	-	-	7	6	-	-
Mucor pusillus	-	-	-	-	-	-	3	7	-	-	-	-	7	-

* Bacteria

Influence of seed mycoflora on seed viability :

With increase in the storage duration there was increase in the incidence of seed mycoflora but decrease in seed viability. The reason for the loss of seed viability are not fully understood but seed ageing and physiological changes in seed during storage are usually ascribed as possible factors responsible for seed deterioration. Though Periera *et al*[8]. opined that the fungi were not the main cause of low seed quality during storage, but our studies have indicated that seed mycoflora has high negative correlation (r = -0.8718) with seed viability and a positive correlation with seed moisture (r = 0.35) during storage (Table 2). This suggested that seed moisture influence seed mycoflora which might be responsible for bringing about certain physiological changes in the seed, leading to loss in seed quality during storage. Vaidehi[1] showed that the storage fungi lowered the quality of maize grains due to the biochemical changes brought about by them. Seed treatment with mancozeb/thiram and storage in polylined bag retained seed viability of soybean 'cvs' P-16 and JS-80-21 for 15 and 13 months after seed harvest respectively, as against 9 months in untreated seeds stored in cloth bags. These fungicides may be acting as protective agents against seed deterioration due to fungal invasion and physiological ageing as a result of which the seed viability was maintained for a comparatively longer period under ambient conditions.

Table 2.– Influence of seed mycoflora on seed viability and seed moisture during storage.

Storage period (in months after seed treatment)	Seed Germination* (%)	Seed Mycoflora* (%)	Seed Miosture* (%)
0	92.0	1.05	7.8
2	86.5	2.4	5.9
4	92.8	3.4	6.6
6	85.3	2.7	9.9
8	50.8	5.9	8.6
10	44.3	4.6	8.9
12	38.3	5.0	8.8
Correlation coefficient (r)	-0.872		0.35

*Average of 20 treatments

Thus, it is inferred from the present studies that both seed treatments and storage containers influence the percent mycoflora associated with soybean seeds during storage for 15 months after seed harvest under ambient conditions. In general, there was an increase in the number of the fungal colonies and decrease in seed viability irrespective of treatments and containers with the advancement in storage period. Of the two storage containers, polylined bag was found superior since the seed stored in it harboured less number of fungal colonies as compared to seed stored in cloth bags and seed treatment with fungicides like mancozeb/thiram prior to storage accorded protection to the seeds during storage and maintained seed viability for longer duration ambient conditions.

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Proline accumulation and lipid peroxidation in wheat seedlings exposed to cadmium toxicity

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Abstract

Imposition of increasing concentrations of cadmium in the form of cadmium chloride on wheat seedlings drastically decreased root growth. An uniform increase in the accumulation of proline, an osmoprotectant was noticed in both leaf and root tissues on 12th day of study. Roots showed more proline accumulation than leaves. Lipid peroxidation measured in terms of malondialdehyde content (MDA) and membrane injury index (%) increased with increasing metal concentration in both leaf and root tissues.

(**Key words :** proline/lipid peroxidation/cadmium/*Triticum aestivum*.)

Introduction

Cadmium is known to affect seed germination, seedling growth and metabolic process in plants. It induces oxidative stress producing reactive oxygen species like O_2, H_2O_2, OH etc. causing high degree of membrane lipid peroxidation and other oxidative injuries[1-5]. The present experiment was carried out to study some of these aspects of Cd toxicity in wheat, a widely cultivated cereal.

Materials and Methods

Dry graded uniform wheat (*Triticum aestivum* L. cv. Sonalika) seeds were taken and surface sterilized with 0.1% $HgCl_2$ for 5-8 minutes and were thoroughly washed and rinsed with distilled water. Seeds were germinated in different petriplates containing moistened whatman No. 1 filter paper and were kept in dark for 2 days at 25 ± 2 °C. On second day germinated seeds were transferred to plastic glasses containing half strength Hoagland nutrient solution and were kept in growth chamber under continuous white light provided by filtered, cool white fluorescent tubes (Philips 36 W TLD) with aphoton flux density of 52 μ molm^{-2}s^{-1}. On seventh day Cd stress treatments were imposed in the form of cadmium chloride solutions (0,0.001,

0.01, 0.1, 1.0 mM). The effect of stress treatments were studied on 12 day old wheat seedling. Proline estimation of both the root and leaf was done as per the method described elsewhere[6]. Homogenates of roots and leaves were prepared with 5% TCA and were taken for extraction and estimation of malondialdehyde (MDA)[7]. For measuring membrane injury index, 200 mg or root and leaf tissues were placed in 15ml deionised water and incubated for 24 h at 25 °C. The electrical conductivity of the medium was measured after 24 h. The tissue with leachate was then autoclaved at 1 kg cm^{-2} pressure. The electrical conductivity of the medium was again measured at room temperature. The membrane injury index was calculated as per the method described elsewhere[8].

Results and Discussion

Increasing concentrations of cadmium decreased the root length growth in 12 day old seedlings as compared to control. However, maximum decrease occurred at 10 mM Cd concentration. Similar inhibition in root elongation has been reported earlier in different plants suggesting the sensitiveness in terms of growth to the metal which seems to be due to a decrease in root absorption area, inactivation or non-induction of key defence machinery[9]. Proline accumulation showed an uniform trend in both leaf and root. However, root cells showed more accumulation of free proline at higher metal concentration. Accumulation of free proline in response to heavy metal exposure seems to be wide spread among plants. The possible reason of accumulation of this osmoprotectant proline in plants under cadmium toxicity is that it is induced by a Cd imposed decrease of the plant water potential and the functional significance would be in the contribution of proline to water balance maintenance[1,3,10,11]. It is also attributed for scavenging of hydroxyl radicals since heavy metal exposure is known to induce oxy-free radical production and lipid peroxidation[12-13].

A drastic increase in lipid peroxidation as evident by malondialdehyde (MDA) accumulation and membrane injury index (%) was recorded with the increase in metal concentrations in tissues of 12d old wheat seedlings. Similar reports are available for different plants[5,14]. Increase in the level of MDA suggeste generations of hydroxyl radicals either by Haber-Weiss of Fenton type reactions[13]. The reason for membrane injury in root tissue than leaf may be due to the heavy metal reaching the shoot is usually lower than the amount present in roots or because of the formation of phytochelatins and binding of Cd to root cell walls[15-16].

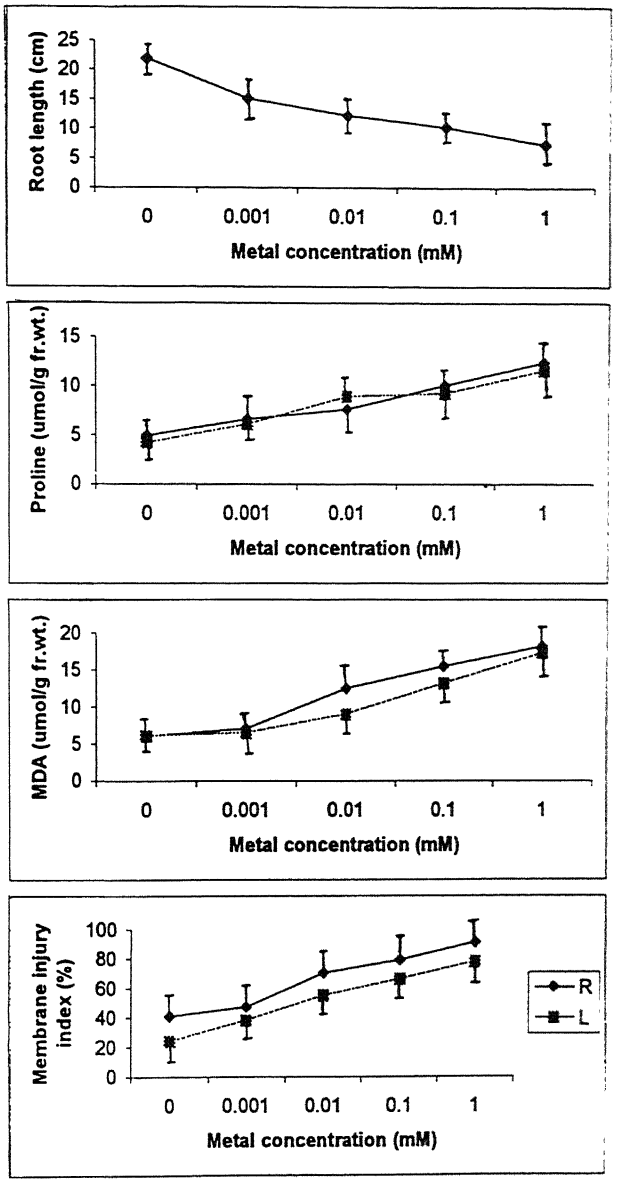

Fig 1.–Changes in root elongation, proline accumulation, malondialdehyde (MDA) content and membrane injury index (%) parameters in root and leaf cells of 12-d old wheat seedlings under cadmium toxicity. Data presented are mean ± SEM.

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Studies on the biological treatment of digested distillery spent wash effluent using mutant strain of *Phanerochaete chrysosporium*

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Abstract

A new mutant strain, *Phanerochaete chrysosporium* GPS-V, showing high decolourization and COD removal capability on digested distillery spent wash effluent (40% v/v) in basal medium was induced from *Phanerochaete chrysosporium* isolated from lagoon soil by enrichment culture technique. In submerged cultures with basal medium containing 40% (w/v) digested spent wash supplemented with additional carbon source, glucose 4% (w/v) and other nutrients, the maximum decolourization of 84% and COD reduction of 91% was achieved at the optimum performance parameters.

(**Key words** : decolourization/digested spent wash/distillery effluent/ *P. chrysosporium*).

Introduction

Anaerobically digested cane molasses spent wash is a dark brown coloured effluent with high chemical oxygen demand (COD), high pollution potential and is bioremediation resistant. The colour is mainly due to the melanoidins, brown polymers which are formed by the Malliard amino-carbonyl reaction[1] and partly due to lignin which are not toxic as such. When these coloured effluents enter rivers or any other surface water system they upset biological activity causing detriment to the aquatic life[2,3]. Disposal of this recalcitrant effluent on land further causes a reduction in soil alkalanity, manganese availability and effecting the ground water system because of the leaching from the soil[4,5]. The treatment of spent wash through physicochemical process is cost intensive and the sludge disposal makes the process further uneconomical[6]. Biological treatment methods under natural conditions such as activated sludge or anaerobic lagooning are intricate[7] and in general, are une-

conomical because of high operational cost and requirement of large land area. Hence, researchers in past have tried bioremediation using various melanoidin and lignin degrading microbes. Microbial decolourization of melanoidin has been detected in fungi. Ohmomo[8] reported a strain of *Aspergillus sp.* showing high decolourization activity by the adsorption phenomena of melanoidin to fungal mycelia. Several other white rot fungi decolourizing melanoidin degradation were also reported[11,12]

In the present communication we report the isolation of a mutant strain of *Phanerochaete chrysosporium*, capable of both bioremediation and decolourization of digested distillery spent wash at higher concentrations.

Materials and Methods

Materials:

The anaerobically digested spent wash was collected from local distillery of Dehradun, India. The effluent was stored in dark at low temperature under refrigeration before being used as substrate for biological treatment.

Organism :

The fungus *Phanerochaete chrysosporium*, was isolated from the lagoon soil by enrichment with digested spent wash[13]. It was maintained on malt extract agar slants, stored at 4 °C and renewed every month.

Mutagenesis and screening :

The mutagenesis of *Phanerochaete chrysosporium* was done according to the standard method[14]. The effluent decolourizing capability of the mutagenised colony was done by measuring the diameter of the halozone. The digested spent wash 10% (v/v) was used as substrate for examining decolourization activity on agar plates. The mineral base medium consist of 2% glucose, 0.5% yeast extract, 0.1% KH_2PO_4, 0.05%, $MgSO_4.7H_2O$ and 2% agar at pH 6.0.

Preparation of the inoculum :

Spore suspension was prepared by adding 10 ml of sterile distilled water to the five day old slants of culture. 5 ml of this culture suspension was transferred to 50 ml

of potato dextrose broth (PDB) and incubated for 72 h. at 30 °C in shake flasks. The growth obtained was used as an inoculum for the microbial treatment of the digested spent wash.

Shake flask decolourization experiments :

All the studies for decolourization of digested spent wash were carried out in a basal medial composed of 10%-80% (v/v) digested spent wash containing 2% glucose, 0.5 NH_4NO_3, 0.1% KH_2PO_4 and 0.05% $MgSO_4.7H_2O$. The pH of the medium was adjusted to 6.0 unless otherwise specified. The experiment was carried out by dispensing 100 ml of basal medium of different concentration of digested spent wash in 250 ml conical flasks, incubated at 30 °C on a rotatory shaker at 150 rpm. At appropriate time flasks were withdrawn and contents were analysed for decolourization, COD, total sugar and total solid contents. The observations reported were measured after 8 days of incubation.

Analytical methods :

The decolourization of digested spent wash was measured as a decrease in optical density at 465 nm of diluted spent wash medium and expressed as percentage decrease in absorbance. Analyses to determine the specific gravity, total reducing sugar, total sugar, total suspended solids, carbon, nitrogen, phosphorus, COD, electrical conductivity etc, for digested effluent were those described in APHA (1989)[15].

Results

The primary treatment of distillery effluent with anaerobic bacteria, recovering fuel gases and removing 80-85% BOD/COD is a most attractive proposal for treatment. The remaining 15-20% BOD/COD is very hard to remove. This is attributable to change in the ratio of biodegradable to non-biodegradable components, end product inhibition or due to the highly complex biorefractory compounds melanoidin which is not easily degraded by the microganisms although it is highly distributed in nature. The chemical components digested spent wash are shown in Table 1.

Isolation of a high decolourizing potential mutant strain of *P. chrysosporium*:

Fig. 1 shows the population distribution of *P. chrysosporium* with respect to decolourization potential in terms of diameters of halozones. The enrichment culture

method generated a high decolourization activity potential strain giving halozone values (diameter) of 8-10 mm. On further mutanization by UV in the presence of enriched basal medium resulting in a mutant designated as *P. chrysosporium* GPS-V which showed higher halozone diameter values than the previously reported isolated strain[13]. The initial activity of fungal strain was clearly evident with the maximum ability of decolourization of 90% and COD reduction of 97% at 1% (v/v) of digested distillery spent wash in basal medium in shake flask experiments. This potential of fungal strain was substantially enhanced by the consecutive optimization of the process.

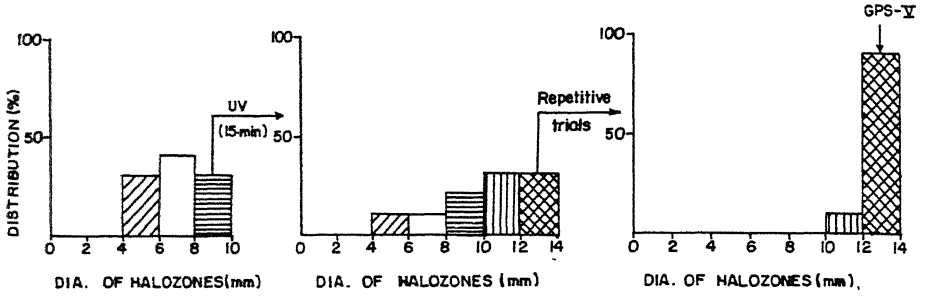

Fig. 1–Selection of mutant strain of *Phanerochaete chrysosporium* for high decolourization activity of digested distillery spent wash after UV mutanization.

Effect of different concentrations of digested spent wash on declourization activity :

The effect of different concentrations of digested spent wash in basal media on decolourization and COD reduction by the fungal culture is shown in Fig. 2. Increased concentration of effluent in basal media reduce both percentage decolourization and COD removal. The potential of the fungal strain was clearly evident upto 40% (v/v) digested spent wash in basal media. Above this concentration level the fungal activity declined rapidly. The inhibitory effect of increasing concentration is likely to be due to the presence of the inhibitory compounds such as phenolics, gallic acid and vanillic acid and additionally due to respective increase in osmotic potential of fermentation medium which hinders nutrient uptake and release of metabolites thus effecting mycelial growth. This shows that substantial dilution is required for spent wash before effective decolorization and COD reduction can be achieved.

Table 1– Chemical composition of Raw and Digested spent wash

Parameters	Raw spent wash	Digested spent was
pH	4.60	7.66
Density	1.14	1.03
Total Solids (g/l)	107.10	32.40
Volatile (g/l)	41.40	16.92
Dissolved solids (g/l)	24.40	7.69
Tatla carbon (g/l)	46.00	8.30
Total nitrogern (g/l)	3.63	1.23
Total phosphorous (g/l)	0.09	0.09
Total sugar (g/l)	29.65	14.30
COD (mg/l)	1,22,000.00	28,600.00
BOD (mg/l)	16,000.00	1,800.00

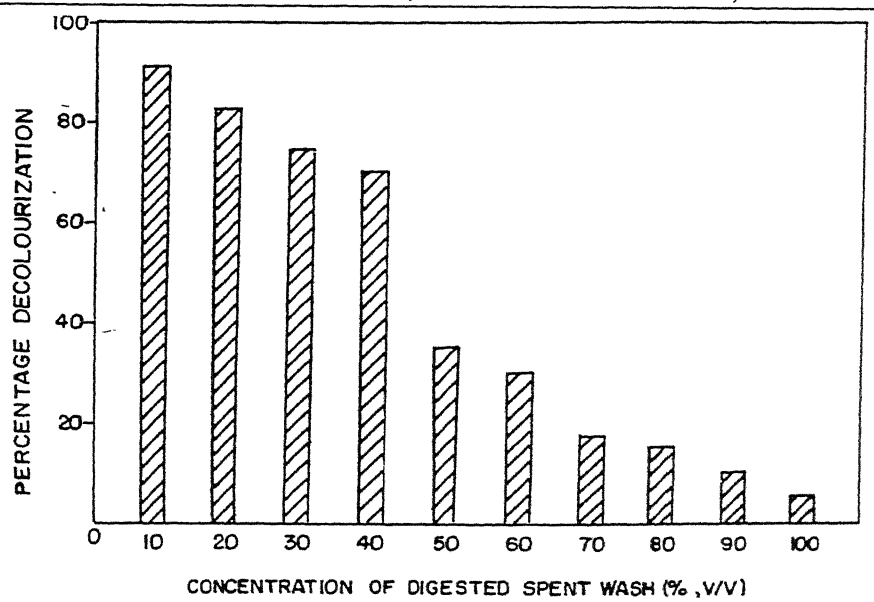

Fig. 2–Percentage declourization by *Phanerochaete chrysosporium* at different concentrations of digested spent wash in basal medium.

Effect of different carbon and nitrogen source on decolourization activity :

The medium containing 40% (v/v) digested spent wash in basal medium was supplemented with different carbon sources in the form of sugars namely glucose,

sucrose, maltose, fructose, mannose, galactose, mannitol, xylose. It was observed that glucose, sucrose and maltose induced the maximum growth and decolourization in 8 days of incubation. Further, the optimal concentration of glucose was determined. It was found that maximum decolourization and COD reduction achieved under the optimum supplementation of glucose 4% (w/v) (Table 2). This shows that digested wash contained little, if any, readily available carbon inspite of its high total sugar content. Therefore, it showed a proportional increase in percentage declourization upto 4% (w/v) glucose. Increasing glucose concentration further did not affect the decolourization. The respective increase in decolourization with uptake of glucose reflects that the components of spent wash does not serve directly as nutrients for fungal growth, instead a secondary metabolite is being released, degrading the melanoidins extracellularly.

Table 2– Effect of added glucose and nitrogen sources on digested spent wash decolourization after 8 days of incubation.

Source (%)	Decolourization (%)	COD removal (%)	Total solids reduction (%)	Total sugar reduction (%)
Glucose (%)				
1	43.23	46.60	48.60	49.96
2	77.93	84.04	87.28	88.96
3	80.53	86.60	90.53	91.66
4	83.88	90.41	94.29	95.66
5	84.00	90.77	94.31	95.85
6	83.50	90.00	93.78	95.04`
NH_4NO_3 (mM/100 ml)				
2.5	74.69	80.51	87.30	90.44
5.0	78.15	84.23	91.05	93.23
7.5	80.13	86.50	93.65	96.20
10.0	80.05	87.25	94.31	96.70
12.5	80.24	87.00	94.04	96.00

Supplementation with various organic & inorganic nitrogen sources also exhibited the marked impact on COD and declourization activity (Table 2). Among the various inorganic nitrogen sources like nitrate, sulphate and chlorides of ammonium, tested, NH_4NO_3 was found to be very effective. Although the maximum activity of decolourization and COD reduction was achieved utilizing yeast extract and peptone. But for the technoeconomic feasibility and further scale-up of the process the use of NH_4NO_3 as the supplemented nitrogen source was better option for the

decolourization and COD reduction in spent wash. Further the optimal concentration of nitrogen source was determined and it was observed that at 0.3% (w/v), NH_4NO_3 i.e. 7.5 mM/100 ml induced the maximum decolourization and COD reduction.

Time Course of decolourization activity :

Under optimal performance parameters, supplementation of glucose, 4% (w/v), NH_4NO_3, 0.3% (w/v), optimal pH 5.0 and temperature 35 °C, the decolourization and COD reduction was observed as a function of time. The results showed the increase in the biomass accumulation with corresponding decrease in the total sugar concentration between 0 to 4 days of incubation. The percentage decolourization and COD reduction in the same period shows a slow trend. The rate of microbial activity of decolourization enhanced between 4 to 8 days of incubation. Lastly, the rate of decolourization slowed down between 8 to 12 days of incubation (Fig. 3). In addition to this, supplemented readily availabe carbon source depleted within 2 days of incubation. This shows that the glucose supplementation was necessary to initiate the growth of fungal culture.

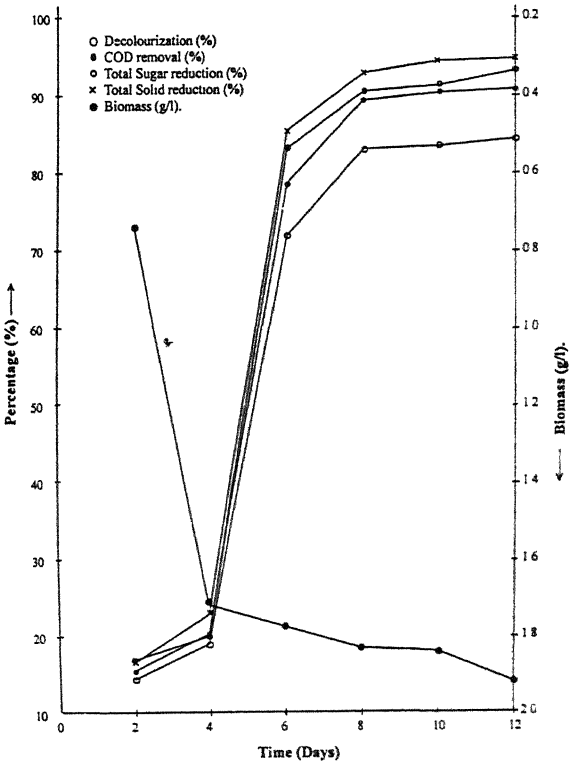

Fig. 3–Time Course of declolourization activity at optimum performance parameters.

Discussion

The present study is an attempt to improve the process of bioremediating the anaerobically digested spent wash effluent, employing an isolated fungal strain *Phanerochaete chrysosporium* GPS-V at concentration of effluent 40% (w/v), in basal medium. The fungal strain, required 6 to 8 days of incubation in attaining the maximal activity of decolourization and COD reduction at optimal performance parameters. It represents optimum growth conditions for the fungal culture which may possibly reflect the optimum activity of enzymes responsible for carrying out the decolourization. Supplementation of the basal media by readily assimilable carbon source was necessary to initiate the growth of fungus. Depletion of the supplemented carbon source in early stages of incubation may drift to refractile carbon of spent wash for further growth. This correlates the fact that the process of decolourization and growth of fungus to a certain extent depends on the uptake of the non-readily available carbon source of the effluent. In addition, glucose supplementation with various organic and inorganic sources shows a marked change on the microbial activity of decolourization. To maintain the optimum fungal nutritional conditions for the maximal activity a definite C:N ratio was maintained by supplementing 0.3% (w/v) NH_4NO_3 to basal media.

The time course data on the microbial activity of decolourization conforms to the classical scheme of Gaden[16] i.e. process of decolourization considered as type II fermentation. The process was characterised by the two rate maxima with regard to growth of the culture and decolourization of the spent wash. The first phase represents the growth of the fungus accompanied by high substrate consumption i.e. readily available carbon and complex carbon of spent wash, with partial decolourization of the effluent. This may be due to the degradation of the smaller molecular weight fractions of melanoidin. The second rate maxima represents the increased rate of decolourization and reduced growth rate of fungal culture. This may be possible by the degradation of the large molecular weight melanoidins in addition to low molecular weight melanoidins by the matured fungal mycelia[8] correlates to the rapid uptake of the sugar and reduction in the solid contents of the digested spent wash. These observations establish that the process of decolourization may occur as a result of the secondary metabolic reactions resulting from secondary metabolites. Whether it is due to sugar oxidase as speculated by *Aoshima et al*, remains to be confirmed[17].

The values of percentage decolourization , COD reduction, total solid reduction and total sugar reduction in the process of treatment of digested spent wash in the

present study are markedly higher than those reported in literature. Further, in our experiments we were able to decolourize upto 81% of the colour of 40% (v/v) digested spent wash in basal medium, which is remarkably higher as compared to the treatment of 12-24% (v/v) digested spent wash in basal medium as reported by earlier workers[3,6,10,12,13].

Thus an attempt of microbial treatment of digested spent wash reported here appears to be quite promising and can be developed into an industrial process.

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Intergeneric hybridization between *Atylosia* and *Cajanus*

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Abstract

Intergeneric hybrids were made between *Cajanus cajan* and three species of *Atylosia* as a female parent. Only 0.6% success could be obtained in the cross between *A. scarabaeides x C. cajan,* 0.8% success was obtained in the cross *A. albicans x C. cajan* and maximum 2.8% in *A. lineata x C.cajan.* Important morphological characters of the three F_1 hybrids were studied at diploid level, have been compared with their respective parents. Meiotic studies revealed the nature and extent of pairing. All the hybrids were semi fertile (51%-72%). In F_2 generation, different plant types were obtained and plant types with more fodder can be used in different range and land situations.

(Key words : *Atylosia/Cajanus/*hybrids/cytology/morphology)

Introduction

Species of *Atylosia* are nutritive fodder legume, well suited in range land situations and also known as wild progenitors of *Cajanus cajan*- an important pulse crop of India. Many workers obtained hybrids between *Atylosia* and *Cajanus cajan* using *Atylosia* as male parent. They could not get success in reciprocal crosses. The presence of reciprocal differences and variation in heterosis for yield in reciprocal crosses has a great bearing in crops like pigeon pea because its uses are manifold both as human food and animal feed. the present study reports the detailed morphological and cytological analysis of successfully obtained three intergeneric hybrids and their segregants.

Materials and Methods

Seeds of materials used were obtained from the Genetic Resources Unit, ICRISAT Hyderabad and the studies were conducted at IGFRI Jhansi. Material

includes *C. cajan* (SNT coll.), *Atylosia albicans* W&A Benth (JM 2337), *A. lineata* (JM 2639), *A. scarabaeoides* (RJW coll), *A. volubilis* (JM 1984), *A. Mollis* (JM 2943), *A. platycarpa* (JM 2873). Techniques of hybridization followed was hand emasculation followed by immediate pollination. All the hybrids were identified on the morphological basis and compared with that of corresponding parents raised in the similar conditions of field and environment. For meiotic studies buds were fixed in propionoalcohol and stained in propiono carmine.

Results

Crosses were attempted in both the directions but success could be obtained only when *Cajanus* was used as a pollen parent. Percent success of crossability was 0.6, 0.8 and 2.7 in *A. scarabaeoides, A. albicans* ans *A. lineata* respectively. Crosses of *Cajanus* with other *Atylosia* species (*A. volubilis, A. platycarpa* and *A. mollis*) were also attempted but success could not be obtained with these species. In these crosses only empty pods having rudimentary seeds were collected which could not germinate.

The hybrid plants exhibited dominant recessive relationship for some qualitative character, vigour for some quantitative characters and intermediate expressions for others (Table 1). In the F_1 hybrid of *A. albicans x C. cajan,* leaves similar to *Cajanus cajan, A. albicans* and intermediate in shape were recorded on the single branch of F_1 hybrid. Pod set percent, ovule fertility and seeds per pod were lower in all the three F_1 intergeneric hybrids, when compared with their parents. In F_2 generation many plants were selected having high fodder values, good seed setting and different growth habits.

During cytological analysis of *C. cajan,* all three species of *Atylosia* and their F_1 hybrids revealed 2n=22 chromosomes (Figs. 1-4). But in the hybrids different degrees of synapsis were observed, which is reflected in Table 2. There was slight reduction in chiasmata per cell and chiasmata per bivalent in all the inter generic hybrids. In the hybrids during late dialkinesis and metaphase-1, hetromorphism was frequently observe (Fig. 3). It is also evident from the Table-1 that there was an increase in the rod bivalents and decrease in the ring bivalents in the hybrids. During meiosis at metaphase-1, one quadirivalent was observed in *A. lineata x C. cajan* hybrids. A range of 0-2 trivalent could be noticed only in *A. scarabaeoides C. cajan* F_1 hybrid. At metaphase-1 univalents were of frequent occurrence. At anaphase-1 and 2 (Table 3) cytological abnormalities included irregular separation of chromosomes, laggards and chromatid bridge which resulted in the formation of micronueclei. In F_2 generation reduction in univalents formation was the prominent cytological feature. In these intergenric hybrids pollen fertility ranged from 62.8% (*A. albicans x C. cajan*) to 77.8% (*A. lineate x C. cajan*).

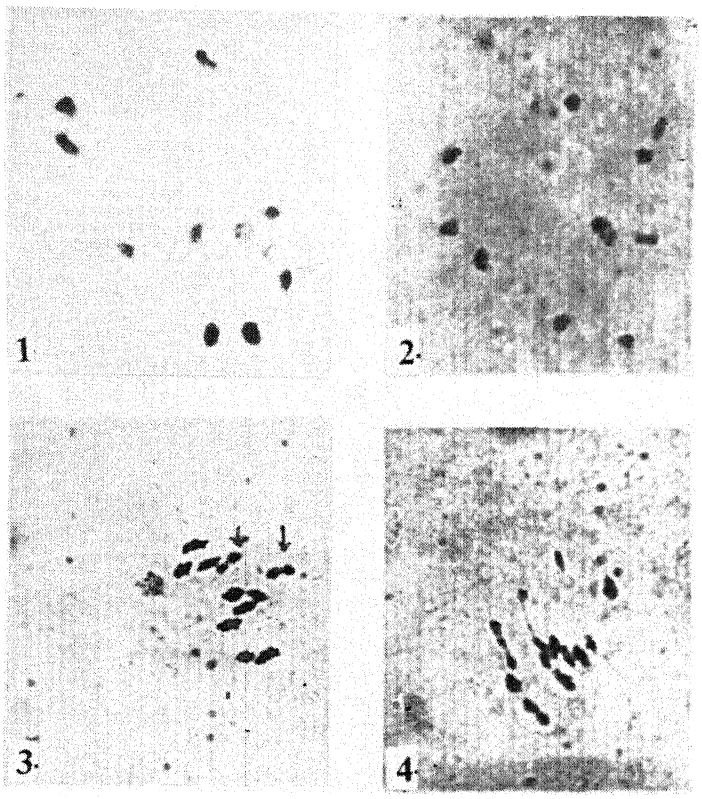

Fig. 1– 11 IIs of *Cajanus cajan* at metaphase-1 (x 1500).

Fig. 2– 11 IIs of *Atylosia lineata* at metaphase-1 (x 1500).

Fig. 3– 11 IIs of *A. lineata* and *Cajanus cajan* F_1 hybrids at metaphase-1 showing two heteromorphic bivalents (x 1500).

Fig. 4– 10 IIs + 2Is at metaphase-1 *A. lineata* × *C. cajan* F_1 hybrid (x 1500).

Table 1–Morphology of *Cajanus, Atylosia spp. and their F_1 hybrids.*

Characters	*C. Cajan (c)*	*A. albicans*	*A. albicans x (c)*	*A. lineata*	*A. lineata x(c)*	*A. scarab.*	*A. scarab. x (c)*
1 Shape of first pair of leaves	Lanceolate	ovate	lanceolate	ovate	lanceolate	ovate	lanceolate
2 Growth habit	Erect shrub	twining shrub	semi erect	erect shrub	erect shrub	herbacious creeper	semi eret
3 Plant height/spread (cm)	103	87	81	96	106	40	65.2
4 Days to flowering	105	134	124	124	134	100	123
5 Central leaflet							
Shape	Oval oblong	obovate	intermediate	lanceolate	lanceolate	obovate	intermediate
Surface	Non hairy	non hairy	non hairy	hairy	hairy	hairy	hairy
Length x breadth (cm)	4.6 x 2.6	4.2 x 3.3	6.5 x 4.3	5.2 x 2.3	5.4 x 2.3	2.5 x 1.4	3.7 x 1.5
Length of petiole (cm)	2.6	4	3.8	2.4	2.8	1.6	1.7
Leaf apices	Emerginate	oval	oval	acute	acute	acute	intermediate
6 Flower							
Colour of standard petal	Pale yellow	brownish yellow	brownish yellow	purplish yellow	purplish yellow	redish yellow	redish yellow
LxB of standard petal (cm)	1.5 x 1.4	1.6 x 1.5	1.8 x 1.6	1.5 x 1.4	1.7 x 1.6	0.8 x 0.51	1.2 x 0.9
Nature of standard petal	Deciduous	persistant	persistant	persistant	persistant	persistant	persistant
7 Pod:							
Colour	Green	green	brown	green	brown	green	brown
LxB (cm)	5.4 x 0.7	1.9 x 0.8	5.0 x 0.9	1.5 x 0.4	2.0 x 0.5	2.1 x 0.50	2.5 x 0.70
Hairiness	Absent	absent	absent	present	present	present	present
Nature of pod	Non shattering	shattering	shattering	shattering	shattering	shattering	shattering
8 Seed - colour	Brown	grey	grey	brown	brown	brown	brown
Seed thickness (cm)	0.002	0.28	0.502	0 3	0.323	0.2	0.302
Seeds/pod	2.2	2.1	1.5	1.82	0 33	2.5	0.6
Strophiole	Absent	present	present	present	present	present	present
9 Pod set %	26.8	61.5	15	64	4.42	64	10
10 Ovule fertility %	85	72	44 8	83	32 8	89	20

Table 2.– Chiasma freqency and chromosome association at metaphase-I in *Cajanu, Atylosia* species.their hybrids.

Plant	Diakinesis			Metaphase										
	Cells studied	Xmata/ cell	Xmata/ bivalent	Cells studied	IV range	IV mean	III range	III mean	II ring range	II ring mean	II rod range	II rod mean	I range	I mean
C. cajan	50	21.16	1.92	50	–	–	–	–		10.3	0-2	0.7	–	–
A.albicans	50	21.36	1.94	50	–	–	–	–	0-11	10.38	0-4	0.6	–	–
A.lineata	50	21.26	1.93	50	–	–	–	–		10.1	0-2	0.9	–	–
A. scarabaeoides	50	21.3	1.93	50	–	–	–	–		10.4	0-2	0.6	–	–
A. albicans x C.cajan	50	17.2	1.62	50	–	–	–	–		8.52	0-8	2.02	0-4	0.47
A. lineata x C.cajan	50	17.9	1.79	50	0-1	0.03	–	–		8.8	0-6	2.34	0-2	0.72
A. scarabaeoides x C.cajan	50	19.34	1.93	50	0-1	0.027	0-2	0.09		0.09	0-5	0.89	0-4	1.27

Table 3–Pollen fertility and chromosome seperation at anaphase 1& 2.

Plant	Anaphase-1				Anaphase-2				Pollen	Size	
	Cells studied	Normal sep.	Laggards	Bridge	Cells studied	Normal sep.	Laggards	Bridge	Fertility	Range	Mean
	No.	%	%	%	No.	%	%	%	%	um	um
C. cajan (c)	82	100	–	–	92	100	–	–	99.2	36-45	42
A. albicans	180	100	–	–	135	100	–	–	99.1	33-39	36
A. lineata	75	100	–	–	60	100	–	–	99.5	36-42	39
A. scarab.	120	100	–	–	200	100	–	–	99.4	30-35	31.5
A. albicans x (c)	70	95.1	2.85	1.42	80	95	3.75	1.25	62.8	33-45	40.5
A. lineata x (c)	60	94.6	3.2	1.66	100	98	2	–	77.8	33-45	43.5
A. scarab.x (c)	60	91.3	3.2	4.93	70	15.14	4.2	–	75.5	30-45	36.6

Discussion

The successful production of hybrids between *Atylosia* and *Cajanus* has shown that the genetic constitution of these genera/ species can produce mature plants. In the present study no success was obtained using *Cajanus cajan* as a female parent. Failure in intergeneric crosses, using, *C. cajan* as a female parent can probably be attributed to the fact that the gene mutaion and selection pressure under domenstication underlying the evolution of the cultivated taxon have resulted in the accumlaiton of the modifiers and differentiation of plasmon in the cultivated taxon *C. cajan*. These changes render *C. cajan* as an unsuccessful seed parent when cross pollinated with *Atylosia* species.

In the crosses of *C. cajan* with *A. platycarpa*. *A. mollis* and *A. volubilis* seedless pods were obtained. Cross failure may exist at the gametic, zygotic or post zygotic levels including hybrid sterility and weakness[1]. In the present study it has been observed that hybrid inviability in these crosses was an active barrier at post fertilization stages. which originated from physiological incompatibility between embryo, endosperm and maternal tissue. This reaction at early stage leads to abortion of young hybrid embryo and at later stage resulted in the formation of partially filled hybrid unviable or rudimentary seeds. Similar conditions *i. e.* pods having partially filled seeds was also reported in the cross between *Phaseolus* species[2].

The F_1 hybrid plants exhibited dominant recessive relationship for some qualitative characters, vigour for some quantitative characters-such as plant height, branching and leaf size and intermediate expressions for others. In case of leaf size, all the F_1 hybrids exceeded to both the parents, as was reported in *C. cajan x A. albicnas* cross[3,4]. In F_2 generation, on a single branch, leaves with different shapes viz, oval oblong, obovate and intermediate shapes were seen in some of the F_2 plants. Variation in leaf morphology accompanied by flowering is a consequence of differential gene expression in different branches and it is likely that this process is temporal event in gene expression. Somatic variation in *C. cajan* was reported to be chimeral which appeared from seedling stage[5]. These workers observed that somatic variation could be mutational in origin but mutation have a low probability of occurrence and are not expected to appear simultaneously in several cells of a tissue but the same is not true of treptions which may occur in all or several cells of a tissue or regions of the body, treptions are the result of a natural stimulus which triggers some regulatory process. In a previous study, in F_2 generation different plant types with more leafiness were recorded[7].

Chromosome behavior at meiosis determines the potentiality of recombinations and it depends upon the number of chiasmata per cell and the position and distribution of chiasmata per bivalent. The factor determining these characteristics are under genetic control and are also related to chromosome size. High chaisma frequency was recorded in the intergeneric hybrids of *Atylosia* species and *Cajanus cajan.* These observations reflects that differentiation in the parental species is primarily at the genetic level which could only be maintained by geographical isolating barriers. However the possibility is there, that these species of *Atylosia* and *C. cajan* may be harbouring cryptic structural differences in their chromosome complement.

Formation of univalents can be attributed to the precocious separation of chromosomes at metaphase-1. This suggests that even the chromosomes of the two parents were apparently similar and non homology seems to exist as revealed by the formation of 0-4 univalents and heteromorphic bivalents at metaphase of these hybrids (Figs. 1-4). Formation of two or three heteromorphic bivalents was reported to be due to possible duplicated segments of chromosomes[7] and such duplication of chromosome segment has been regarded to give opportunities for the differentiation of genus with new function and establishment of lateral heterozygosity[8]. The fact that different chromosomes of *Cajanus* exhibited heteromorphism in different hybrids of *Atylosia* suggests that these species have followed different evolutionary path way for a considerable period.

There is possibilities of selection of different plant types having more fodder potentialities with good seed setting.

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